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BIOASSAY OF ADRENOCORTICOTROPIC HORMONE[1]

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FROM the time that Smith (1930) showed that atrophy of the adrenal cortex followed hypophysectomy of the rat, and showed that pituitary implants would reinstate the adrenal cortex, a method was available for measuring the adrenocorticotropic hormone content of pituitary extracts. Although repair of the adrenal of the hypophysectomized rat has been used in this laboratory since 1932 as a measure of the adrenocorticotropic content of various extracts, a unit has not been proposed. Collip and co-workers (1933) recommended removal of one adrenal from the hypophysectomized rat as a control, the weight of which was to be compared with the weight of the remaining adrenal after the animal was subjected to the influence of injected adrenal cortex-stimulating substances.

Moon (1937) proposed a unit, employing the normal rat, defined as a 50 per cent increase in the weight of the adrenals of 21-day-old male rats injected intraperitoneally once daily for 3 days and killed after 72 hours. A second method for measuring adrenocorticotropic activity suggested by Moon (1940) was the increase in weight of the adrenals of 4-day-old suckling rats. The former test is less sensitive than the one based on the use of the hypophysectomized rat; the latter test has the disadvantage that it is difficult to inject crude fractions or those containing toxic substances into such young animals so that the method would be adaptable only for purified preparations.

The response of the bird's adrenal has also been considered as a method of testing for adrenocorticotropic hormone. The adrenal of the immature pigeon, the test animal used in the standardization of lactogenic and thyrotropic hormones, has proved unreactive to adrenocorticotropic extracts, both on the basis of adrenal weight increment

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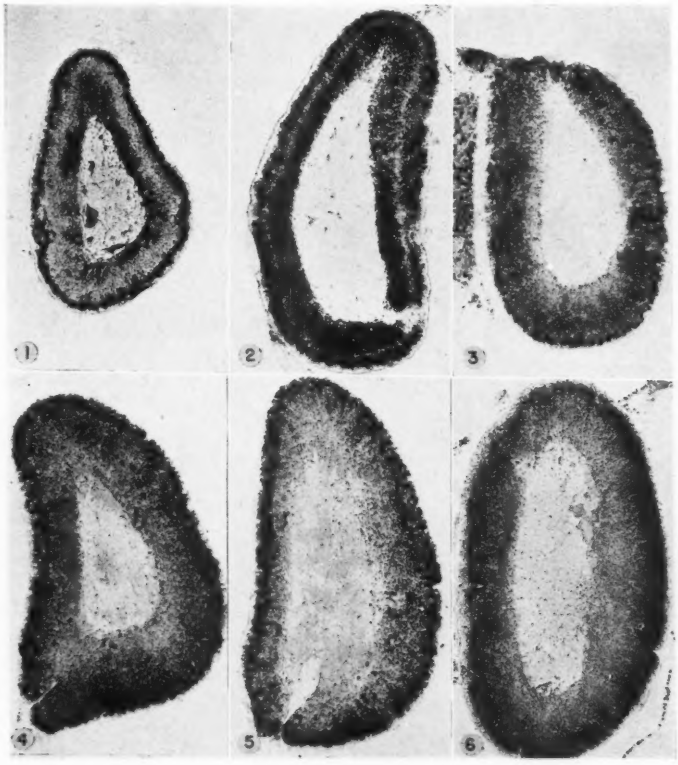
and histological changes (E. M. McNeel, this laboratory, unpublished). The response of the 2-day-old chick adrenal has been proposed as a measure of adrenocorticotropic activity (Bates, Riddle and Miller, 1940). We have found this test likewise insensitive.

In this paper, two methods will be presented for standardization of the adrenocorticotropic hormone; *a*) a method based on partial repair of the atrophic adrenals of hypophysectomized rats, and *b*) the maintenance of normal adrenal weight and morphology by injection immediately after hypophysectomy. Such a maintenance method has also been suggested recently by Astwood and Tyslowitz (1942). Sayers, White and Long (1943) have used standardization methods based both on maintenance and repair.

ADRENAL REPAIR

The method of assay which has been adopted in this laboratory, based on beginning repair of the adrenals of hypophysectomized rats, is as follows. Female rats, 26 to 28 days of age were hypophysectomized and the adrenals allowed to regress for 14 days, at which time injections were instituted and continued for 4 days, once daily intraperitoneally, followed by autopsy 96 hours after the first injection. The minimum detectable effect on the adrenal cortex, often observable before significant increase in size occurred, was a change in the amount and distribution of the lipid droplets. For microscopic examination, the adrenals were fixed in formol, cut as frozen sections, and stained with Sudan Orange. The sections were of uniform thickness, about 12 micra. An effort was made to keep the stain uniform; a light nuclear stain with hematoxylin proved useful.

The adrenal cortex of the hypophysectomized control, 18 days after the operation, was used as the standard of reference (fig. 1). After this postoperative interval the adrenal was reduced in weight from 18 to 9 mg., the reduction being due to decrease in the cortex. The zona fasciculata was greatly reduced, and there was an almost complete disappearance of the zona reticularis; the zona glomerulosa was unchanged or slightly wider. There was in some instances a disruption of the orderly arrangement of zones. The lipid droplets were larger and more irregular in size, and stained a deeper orange than in the normal adrenal. (The lipid droplets in the adrenal of the normal rat are fine, evenly distributed, and stain a light golden color). This difference in staining reaction in the hypophysectomized animal's adrenal may be due only to size of lipid particles or may be due in part to changes in the types of lipids present. The distribution of the lipids in the adrenal cortex of the hypophysectomized control differed from that found in the normal female in a still more important respect, namely, lipid almost completely disappeared from the subglomerular portion of the zona fasciculata giving rise to the so-called subglomerular sudanophobic zone.



Repair of the adrenal cortex of hypophysectomized rats by adrenocorticotropic hormone. Female rats 26–28 days of age at operation, 14 days postoperative at beginning of injections. Various doses given as single daily intraperitoneal injections for 4 days. Sudan orange stain. ×38.

FIG. 1. Hypophysectomized control, uninjected, 18 days postoperative. Depletion of lipid in narrow zona fasciculata (sudanophobic zone). Lipid droplets coarse and deep-staining in zona glomerulosa and zona reticularis. Weight of adrenals, 9 mg. (average adrenal weight at operation 18 mg.).

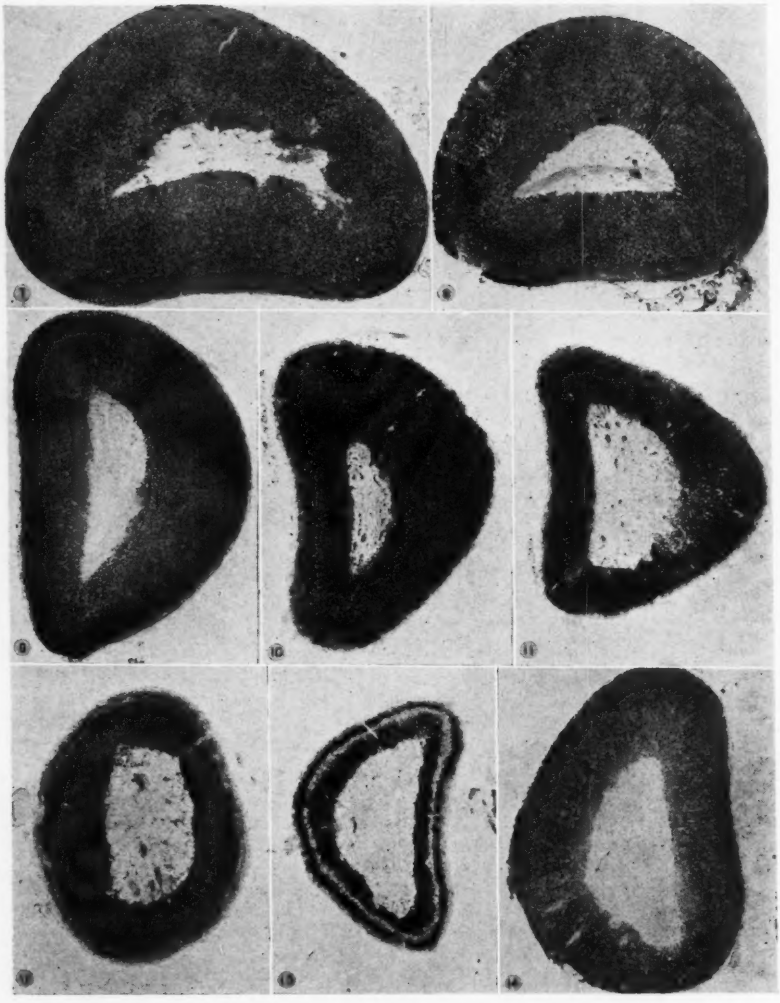
FIG. 2. Total dose 0.025 mg. Cortex not significantly wider than in control, but some infiltration of lipid into fasciculata narrowing the sudanophobic zone. Lipid droplets somewhat finer; therefore lighter staining than in control. Reaction +1. Weight of adrenals, 12 mg.

FIG. 3. Total dose 0.05 mg. Cortex only slightly wider than in control. Obliteration of subglomerular sudanophobic zone. Lipid distribution almost uniform. Reappearance of zona reticularis. Reaction +2. Weight of adrenals, 11 mg.

FIG. 4. Total dose 0.1 mg. Slight increase in width of cortex. Reaction +2. Weight of adrenals, 12 mg.

FIG. 5. Total dose 0.2 mg. Slight increase in width of cortex. +2 reaction. Weight of adrenals, 16 mg.

FIG. 6. Total dose 1 mg. Further development of cortex, +3 reaction. Weight of adrenals, 18 mg.



Maintenance of the adrenals of male rats hypophysectomized at 40 days of age and injected twice daily, intraperitoneally, beginning the day of operation, for 15 days with various dosage levels of adrenocorticotropic hormone. Sudan orange stain. ×38.

FIG. 7. Daily dose, 2 mg. Weight of adrenals 66 mg.; larger than in 41-day-old control (fig. 14); lipid distribution in cortex uniform, droplets fine; hypertrophy of cortical cells; clear vacuoles, unstained by Sudan orange, especially in outer third of cortex.

FIG. 8. Daily dose, 0.5 mg. Weight of adrenals 42 mg. Hypertrophy, as in fig. 7.

FIG. 9. Daily dose, 0.2 mg. Weight of adrenals 37 mg. Maintenance.

FIG. 10. Daily dose, 0.1 mg. Weight of adrenals 26 mg. Maintenance. Adrenals shown in fig. 9 and 10 are about the size of those of normal rats at time of hypophysectomy (fig. 14, weight 34 mg.). The cortical cells are not so noticeably enlarged

The changes observed in the adrenals of hypophysectomized rats at the lowest effective doses of adrenocorticotropic hormone were as follows. Individual cells constituting the zona fasciculata and glomerulosa became larger and therefore more distinct when examined with low magnification. Lipid droplets were finer and more evenly distributed; the subglomerular sudanophobic zone became partially infiltrated with lipid but was still present, although it was narrower. Some increase in width of the fasciculata and reappearance of the zona reticularis may have occurred. This minimal response, in which the most important criterion was a beginning redistribution of lipid, was designated as a +1 reaction (fig. 2).

At higher dosage levels, these changes became more pronounced. Paralleling the decrease in size of the lipid droplets there was further redistribution of the lipid, leading to the disappearance of the sudanophobic or lipid-free subglomerular zone. When this zone was obliterated by infiltration of lipid so that a subglomerular zone could no longer be distinguished, the reaction was called a +2 reaction (fig. 3, 4, 5). Some increase in adrenal weight and a recognizable increase in width of the cortex were consistently present when lipid redistribution had reached this stage. The increase in weight of the adrenal during this brief period of repair was, however, slight and of no confirmatory value to the histological changes.

The microscopic changes just described did not follow one another in an invariable sequence. It can be stated, however, that when the doses given were sufficiently large to cause obliteration of the subglomerular sudanophobic zone, the lipid droplets were found to be definitely finer and more homogeneous, and some increase in the width

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as at the higher dosage levels, being about normal size. Clear vacuoles are not seen. Some animals in these two groups showed beginning depletion of lipid in the subglomerular zone, about comparable to that seen in the adrenal of the normal male rat.

FIG. 11. Daily dose, 0.05 mg. Weight of adrenals, 18 mg. Although below normal control weight, the adrenals are essentially normal in structure.

FIG. 12. Daily dose, 0.02 mg. Weight of adrenals, 20 mg. Although the same weight as those in the preceding figure, the adrenals show structural evidence of less stimulation. Lipid distribution is no longer uniform, a subglomerular lipid-free zone is present, which is wider than in the normal male and more like that in the adrenal depleted after hypophysectomy. The lipid in the outer part of the glomerulosa is also depleted. The zona fasciculata and reticularis are still quite wide and lipid droplets are still fine.

FIG. 13. Hypophysectomized control, uninjected; age at operation, 40 days, 15 days postoperative. Weight of adrenals, 16 mg. The cortex is markedly reduced in width due to the disappearance of most of the zona fasciculata and reticularis. The lipid droplets in the glomerulosa and the remaining portions of the fasciculata and reticularis contain heavy deposits of lipid in large droplets. A distinct, fairly wide subglomerular sudanophobic zone is present.

FIG. 14. Normal control, uninjected, age 41 days. Weight of adrenals, 34 mg. The lipid is evenly distributed through all three zones and occurs in fine droplets so that the cortex stains lightly with sudan orange. The narrow sublgomerular sudanophobic zone, frequently but not invariably present in the adrenal of the normal male rat, is not evident in this case.

of the cortex usually had occurred. When all of these changes were present, and there had also been a considerable increase in width of cortex, together with a reëstablishment of the normal orderly arrangement of zones, the reaction was called a +3 reaction (fig. 6). A total dose in 4 days of 0.010 to 0.025 mg. of pure adrenocorticotropic hormone (Li, Simpson and Evans, 1942; 1943) gave a +1 reaction, 0.025 to 0.050 mg. a +2 reaction, 0.10 to 0.500 mg. a +3 reaction. The unit was defined as the minimum amount of hormone necessary to cause a detectable change in the histological pattern of adrenals of hypophysectomized rats under the conditions described. Based on this definition, the potency of pure sheep adrenocorticotropic hormone was 100 units per milligram.

The age of the female rat chosen for this test depended somewhat upon convenience in this laboratory as this is the age of the rats used in assay of the gonadotropic, growth and thryotropic hormones; rats immediately after weaning, 21-days-old, are satisfactory. Male rats could be used in the same way as described for females.

ADRENAL MAINTENANCE

The second method for standardization of adrenocorticotropic hormone, based on maintenance of the adrenals by institution of injections immediately after hypophysectomy, had several advantages over the method based on repair after atrophy. In the latter test the histological changes noted might occur in the absence of appreciable increase in size of the adrenal, so that at lower dosage levels adrenal weight changes had little confirmatory value. If, on the other hand, injections were begun immediately after hypophysectomy, the marked loss in weight of the adrenals characteristic of this early postoperative period could be prevented by administration of the hormone. Immature or mature rats were used in the maintenance test. Male and female rats as young as 21 days of age proved satisfactory. However, slightly older rats, 40 to 90 days of age, had the advantage that the adrenals were larger at operation and a greater adrenal weight loss occurred during the postoperative period in uninjected controls. Thus, a wider range was available within which differences in weight response to graded dosage could be measured.

In maintenance studies with such older rats it was preferable to use males rather than females in order to avoid cyclic changes in the adrenal. The maintenance test is as follows. Male rats 40 days old were hypophysectomized and injected intraperitoneally, daily (except Sunday) from the day of operation for 15 days (13 injections). A unit was defined as the daily dose which maintained the pre-operative adrenal weight under these conditions. Table 1 shows the response of the adrenals to graded doses of the hormone. It will be seen that a daily dose somewhat in excess of 0.20 mg. gave complete maintenance

of adrenal weight. The pure hormone therefore contained approximately 5 such units per milligram.

This unit was based on a single daily injection of the hormone. Multiple daily injections produced better adrenal response to a given dose. The single daily injection was adopted purely as a matter of convenience. As will be seen in figures 7 to 14, a unit based on two

TABLE 1. BIOASSAY OF ADRENOCORTICOTROPIC HORMONE IN 40-DAY-OLD HYPOPHYSECTOMIZED MALE RATS (MAINTENANCE TEST)

Daily Dose[1]	No. of Rats	Average Body Wt. at Autopsy	Adrenal Wt. Aver.	Thymus Wt. Aver.
mg.		*gm.*	*mg.*	*mg.*
1.0	10	107.8	40.1 ±0.8[2]	46.8
0.20	15	117.4	25.1 ±0.9	130.8
0.10	15	117.9	22.3 ±1.0	193.7
0.05	25	118.6	19.5 ±1.0	190.8
0.02	14	107.8	15.6 ±0.7	201.2
0.00	19	127.5	12.0 ±0.5	222.4
Normal Control 40 days	171	130.0	28.3 ±0.41	363.4

[1] A single injection daily.
[2] The ± values are the standard error.

injections daily would be 0.1 mg. (fig. 10) instead of 0.2 mg. as defined. These figures, depicting the adrenal morphology throughout a wide range of dosages, show clearly the normal morphology of the adrenals of animals receiving the unit dose based on weight maintenance.[2]

SUMMARY

Two methods have been proposed for standardizing the adrenocorticotropic potency of pituitary extracts. The units are defined as a) the total dose in milligrams necessary to cause beginning adrenal repair (redistribution of cortical lipid) in female rats 26 to 28 days old at hypophysectomy and 14 days postoperative at the beginning of the 4-day injection period. b) The daily dose in milligrams necessary to

[2] The size of the adrenal and distribution of lipids might not be considered a specific test for adrenocorticotropic hormone. This is certainly true for normal rats, as many adverse conditions cause enlargement of the adrenals in such animals, probably through their effect on the pituitary. It should be stressed that the changes in adrenals reported here were all obtained in the absence of the pituitary. Greep, *et al.* (1942) reported changes in the adrenal resulting from injection of lactogenic hormone. In our experience lactogenic hormone is extremely difficult to free from the last trace of adrenocorticotropic hormone but the pure substance shows no maintenance of the adrenal of hypophysectomized rats in doses of 1 mg. daily for 15 days. Golla and Reiss (1942) have found that pregnant mare's serum gonadotropic preparations enlarge the adrenals of hypophysectomized rats but find the adrenotropic factor more heat stable than the gonadotropic factor present in the mare serum. Their work may therefore be considered to indicate the presence of adrenal-stimulating substance in body fluids rather than as indicating the non-specificity of the test here described.

maintain the pre-operative adrenal weight for 15 days, in male rats hypophysectomized at 40 days of age.

REFERENCES

ASTWOOD, E. B., AND R. TYSLOWITZ: *Fed. Proc.* 1: 4. 1942.
BATES, R. W., R. RIDDLE AND R. A. MILLER: *Endocrinology* 27: 781. 1940.
COLLIP, J. B., E. M. ANDERSON AND D. L. THOMSON: *Lancet* 1: 1208. 1933.
GOLLA, Y. M. L., AND M. REISS: *J. Endocrinol.* 3: 5. 1942.
GREEP, R. O., B. F. CHOW and H. B. VAN DYKE: *Fed. Proc.* 1: 33. 1942.
LI, C. H., H. M. EVANS AND M. E. SIMPSON: *J. Biol. Chem.* 149: 413. 1943.
LI, C. H., M. E. SIMPSON AND H. M. EVANS: *Science* 96: 450. 1942.
MOON, H. D.: *Proc. Soc. Exper. Biol. & Med.* 35: 649. 1937.
MOON, H. D.: *Proc. Soc. Exper. Biol. & Med.* 43: 42. 1940.
SAYERS, G., A. WHITE AND C. N. H. LONG: *Proc. Soc. Exper. Biol. & Med.* 52: 199. 1943.
SMITH, P. E.: *Am. J. Anat.* 45: 205. 1930.

FACTORS AFFECTING THE ACTION OF ANTI-GONADOTROPIC SERA IN IMMATURE RATS[1]

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WHILE investigating the biological and chemical characteristics of antigonadotropic substances it was observed that the results obtained were determined to a large extent by the degree of purity and source of gonadotropic hormone preparations, and the method of injecting them and the antisubstance. Thus it was found that an antigonadotropic serum prevented the activity of certain gonadotropins only when injected separately into rats while the actions of other hormone preparations could be inhibited by injecting them either separately or in combination with an antigonadotropic serum. The object of this report is to present results of a study concerned with the effect of injection procedures on the inhibition of different gonadotropins and gonadotropic preparations of varying purity, by antigonadotropic sera.

MATERIALS AND METHODS

The antigonadotropic sera used in this study were developed in goats and rabbits by injecting them with an aqueous extract of whole sheep pituitary powder which was made by extracting the powder with water, precipitating the active material with acetone, and extracting the acetone precipitate with water followed by supercentrifugation.

The goats were injected 5 times weekly over a period of 30 weeks with 250 mg. equivalent of this supercentrifuged aqueous extract of sheep pituitary powder. Blood was collected from these goats at varied intervals during the last 16 weeks of the injection period. The rabbits were injected daily with 250 mg. equivalent of a supercentrifuged aqueous extract of sheep pituitary glands, for a period of 8 weeks. At the end of this time and at varying intervals thereafter the rabbits were bled while the administration of hormone was continued.

The blood containing the antigonadotropic substance was obtained at intervals from the rabbits by bleeding from the heart, and from the jugular veins of the goats. The blood upon clotting was centrifuged and the serum removed and stored in the frozen condition. The various lots of sera were finally combined and by this method two large quantities of rabbit serum and another of goat serum were obtained. These sera were kept frozen except when aliquots were removed for testing.

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The gonadotropins used in testing the antisheep-pituitary sera from rabbits and goats were aqueous extracts of sheep, hog, cow, horse, human and rat pituitary powders. As gonadotropin of cow pituitary gland is not effective in the rat in the dose used it was necessary to augment it by adding 6 mg. of hemin dissolved in dilute alkali, to each 500 mg.[2] of cow pituitary extract (McShan and Meyer, 1941). The follicle-stimulating preparation was made from sheep pituitary powder by the method of McShan and Meyer (1940). Untreated pregnant mares' serum, and purified gonadotropin of human pregnancy urine prepared by the method of Gurin, Bachman and Wilson (1939) were also used in testing the antisera.

It became of interest during this investigation to determine whether the degree of purity of sheep pituitary extracts influenced the action of antigonadotropic sera when administered by the combined as compared to the separate injection procedure. For this purpose a method was developed for preparing a highly purified sheep gonadotropin having both luteinizing and follicle-stimulating activities. The process of purification was such that it was possible to test four different fractions each representing a progressive stage in purification. The method is briefly described as follows: Fraction A, the aqueous extract of sheep pituitary powder, was adjusted to pH 5 by dialysis against pH 4 acetate buffer and a pH 5 soluble fraction B was separated from the resulting precipitate by centrifuging. Fraction B was then adjusted to pH 4 by dialysis against the acetate buffer and a pH 4 soluble fraction C was recovered by centrifuging. Fraction C was dialyzed against distilled water and fraction F was obtained from the soluble portion by the use of alcohol and by dialysis. This procedure by which extract A was used in the preparation of fractions B, C and F is given in detail in a report which is in press.

A typical example of the progressive decrease in the solid content of these fractions per gram equivalent of original pituitary powder was 88.8 mg., 70.9 mg., 24 mg., and 5.1 mg., respectively, for extracts A, fractions B and C, and preparation F. Comparison of these solids with the biological activity of each of these fractions contained in table 2 indicates that this degree of purification was effected without significant loss in activity.

Twenty-one-day-old female rats of the Sprague-Dawley strain were used in testing the antigonadotropic sera. Two methods were used: *a*) The gonadotropic extract and the antiserum were mixed *in vitro* and 0.5-cc. aliquots injected subcutaneously on the afternoon of the first day and twice daily with the same quantity for the next 4 days. *b*) The antiserum and gonadotropic preparation were not mixed, but each was injected subcutaneously in different regions of the rat; 0.5 cc. of the gonadotropic preparation and 0.25 cc. of the antiserum were injected according to the schedule described for the combined injections. Autopsy of the rats was done on the morning of the sixth day, and the ovaries were removed and rapidly weighed to the nearest milligram.

The average ovarian weights of uninjected rats at 26 days of age is approximately 13 mg.

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[2] Indicates in all cases milligrams equivalent of original pituitary powder.

RESULTS AND DISCUSSION

The data in table 1 show the difference in effectiveness of antisheep-pituitary sera of rabbits and goats when tested against sheep and hog gonadotropins by the combined as compared to the separate injection method. The ovaries of rats given 125 mg. of sheep pituitary extract, SAP26A, had an average weight of 81 mg. while those given the same quantity of extract and 1 cc. of antisheep-pituitary serum from the rabbit by the separate injection method had average ovarian weights of 14 mg. However, when the same amounts of extract and serum were combined before injection, the average ovarian

TABLE 1. ACTION OF ANTISHEEP SERA FROM RABBITS AND GOATS ON SHEEP AND HOG PITUITARY GONADOTROPINS AS AFFECTED BY SEPARATE AND COMBINED INJECTIONS

Hormone Preparation		Antisera		No. of Rats	Kind of Inj.	Weight of Ovaries
Kind	Dose	Kind	Dose			
	mg. eq.		*cc.*			*mg.*
Sheep SAP26A	125	—		12		81
Sheep SAP26A	125	Antisheep-pituitary rabbit PRS26	1.0	3	S	14
Sheep SAP26A	125	Antisheep-pituitary rabbit PRS26	1.0	3	C	85
Sheep SAP26A	125	Antisheep-pituitary rabbit PRS26	4.0	3	C	15
Sheep SAP26	150	—		12		76
Sheep SAP26	150	Antisheep-pituitary rabbit PRS26	0.5	3	S	29
Sheep SAP26	150	Antisheep-pituitary rabbit PRS26	0.75	3	S	21
Sheep SAP26	150	Antisheep-pituitary rabbit PRS26	1.0	9	S	16
Sheep SAP26	150	Antisheep-pituitary rabbit PRS26	1.0	3	C	120
Sheep SAP26A	125	Antisheep-pituitary goat PBGS1RW1	0.25	3	C	38
Sheep SAP26A	125	Antisheep-pituitary goat PBGS1RW1	0.25	3	S	13
Sheep SAP26A	125	Antisheep-pituitary goat PBGS1RW1	0.5	3	C	13
Sheep SAP26A	125	Antisheep-pituitary goat PBGS1RW1	0.5	3	S	11
Sheep SAP26A	125	Antisheep-pituitary goat PBGS1RW1	1.0	6	C	16
Sheep SAP26A	125	Antisheep-pituitary goat PBGS1RW1	1.0	3	S	10
Hog 5HAP16	275	—		12		65
Hog 5HAP16	275	Antisheep-pituitary goat PBGS1RW1	1.0	3	S	10
Hog 5HAP16	275	Antisheep-pituitary goat PBGS1RW1	1.0	3	C	82
Hog 5HAP16	275	Antisheep-pituitary goat PBGS1RW1	4.0	3	C	16
Hog 5HAP16	275	Antisheep-pituitary rabbit PRS26	1.0	6	S	35
Hog 5HAP16	275	Antisheep-pituitary rabbit PRS26	1.0	6	C	129
Hog 5HAP16	275	Antisheep-pituitary rabbit PRS26	2.0	3	S	16
Hog 5HAP16	275	Antisheep-pituitary rabbit PRS26	2.0	3	C	102
Hog 5HAP16	275	Antisheep-pituitary rabbit PRS26	4.0	3	C	152
Hog 5HAP16	100	—		6		39
Hog 5HAP16	100	Antisheep-pituitary rabbit PRS26	1	3	S	14
Hog 5HAP16	100	Antisheep-pituitary rabbit PRS26	1	6	C	74
Hog 5HAP16	100	Antisheep-pituitary rabbit PRS26	4	3	C	14

weight was 85 mg. If the quantity of antisheep-pituitary serum of the rabbit was increased to 4 cc. and combined with 125 mg. of sheep anterior pituitary extract before injection, the action of the gonadotropin was inhibited. Results of similar kind were obtained when antisheep-pituitary serum from the rabbit was given by the separate injection method in 0.5, 0.75 and 1 cc. doses with 150 mg. of another sheep-pituitary extract SAP26, as compared to 1 cc. of antiserum given with 150 mg. of sheep extract by the combined injection procedure. (Extract SAP26 was not as active as SAP26A.) The latter method did not inhibit the gonadotropic activity of the extract, but caused an increase in ovarian weight greater than that produced by 150 mg. when given alone. These data demonstrate that rabbit serum containing an

antisheep-pituitary substance does not as effectively inhibit the action of sheep pituitary gonadotropin when it is given by the combined injection method as it does when administered separately. The same conclusion is supported by the data obtained with antisheep-pituitary serum from the goat which was more active than the rabbit serum, as 0.25 cc. of the former completely inhibited 125 mg. of extract SAP26A, when given by separate injections, but 1 cc. of rabbit serum was required to neutralize the action of 150 mg. of SAP26. All doses of the antisheep-pituitary serum of the goat in excess of 0.25 cc. inhibited sheep gonadotropin when given either by the separate or combined injection method.

Attention is directed to the relative ineffectiveness of antisheep-pituitary serum from the rabbit in neutralizing the action of hog pituitary extract as compared to sheep gonadotropin. One cc. of antisheep-pituitary serum from the rabbit when injected separately completely inhibited the action of 125 mg. of sheep extract, but not completely the action of 275 mg. of hog extract although 2 cc. did. Four cc. of antisheep-pituitary serum from the rabbit when given by the combined injection method neutralized the gonadotropic effect of 125 mg. of sheep extract but not that of 275 mg. of hog (table 1).

The data presented indicate that the potency of an antisubstance influences the results obtained by the combined injection method. The antisheep-pituitary serum from the goat was decidedly more potent than the rabbit serum for when administered separately 0.25 cc. of the former neutralized the hormone effect while 1 cc. of the latter was necessary. Likewise, by the combined injection method 0.5 cc. of the goat serum completely inhibited the sheep gonadotropin but it took 4 cc. of the rabbit antisera to have a similar effect. A comparison of the effects of the antisheep-pituitary sera from goats and rabbits on the action of the hog pituitary extract also supports this conclusion.

The data given in table 2 show the effect of the degree of purity of the gonadotropic extract on the inhibitory action of antisheep-pituitary serum from the rabbit. Extracts 74 A, B, C and F are respectively the aqueous extract of sheep pituitary powder, the pH 5-soluble fraction, the pH 4-soluble fraction and the purified fraction. The least and most purified fractions, A and F, respectively, were completely prevented from acting by 1 cc. of antiserum administered by the separate injection method. Fractions A and B, the less purified, were only partially inhibited by the antiserum when given by the combined injection procedure, but fractions C and F which were more purified were completely inhibited when administered by this method. This experiment was repeated in part using extracts 78 A and F with essentially the same results.

The data of table 3 show the results that were obtained when antisheep-pituitary rabbit serum was tested by both separate and

combined injections with different gonadotropins. The rabbit antiserum when given separately in 1 cc. doses inhibited the action of sheep, horse, human, rat and cow pituitary glands, follicle-stimulating hormone and pregnant mares' serum preparations, and that of hog when given in a 2 cc. dose. Like quantities of antiserum, when combined before injection into rats, inhibited all the preparations except sheep, hog and cow.

The results shown in tables 2 and 3 can be correlated with the amounts of inert material found in the various preparations. Frac-

TABLE 2. RELATION OF DEGREE OF PURITY OF SHEEP PITUITARY GONADOTROPIN TO INHIBITION BY ANTISHEEP-PITUITARY SERUM FROM RABBITS

Hormone Preparation[1]		Antisera		No. of Rats	Kind of Inj.	Weight of Ovaries
No.	Kind of fraction	No.	Dose			
			cc.			*mg.*
74A	Aqueous extract	—	—	11	—	110
74A	Aqueous extract	PRS26	1.0	6	C	63
74A	Aqueous extract	PRS26	1.0	3	S	11
78A	Aqueous extract	—	—	3	—	72
78A	Aqueous extract	PRS79	1.0	3	C	120
78A	Aqueous extract	PRS79	1.0	3	S	16
74B	pH 5 sol. of A	—	—	6	—	122
74B	pH 5 sol. of A	PRS26	1.0	6	C	61
74C	pH 4 sol. of B	—	—	3	—	103
74C	pH 4 sol. of B	PRS26	1.0	3	C	14
F74	Purified from C	—	—	6	—	127
F74	Purified from C	PRS26	1.0	3	C	12
F74	Purified from C	PRS26	1.0	3	S	11
F78	Purified from C	—	—	6	—	134
F78	Purified from C	PRS79	1.0	3	C	13
F78	Purified from C	PRS79	1.0	3	S	11

[1] Dosage, 100 mg.eq.

tions A and B, and hog, cow and sheep extracts contained much solid material and were completely inhibited only by the separate injection method, but all the preparations which contained much smaller amounts of inert material were effectively inhibited by either injection procedure.

It is suggested that when an antigonadotropic serum is combined *in vitro* with a pituitary gonadotropic extract, the inert material of the extract combines rapidly with part of the specific antigonadotropic substance, thus leaving an insufficient amount to act on the hormone. However, when injected separately, combination of the inert material with the specific antigonadotropic substance occurs very slowly or not at all, so that a sufficient quantity of antisubstance is present to neutralize the hormone. The addition of more or a stronger antigonadotropic serum can compensate for the inert material in the extract

TABLE 3. ACTION OF ANTISHEEP-PITUITARY SERUM FROM RABBITS ON GONADOTROPINS FROM VARIOUS SOURCES AS AFFECTED BY SEPARATE AND COMBINED INJECTIONS

Tests of Antisera Against Gonadotropins					
Hormone Preparation		Anti-sera	No. of	Kind of	Weight of
Kind	Dose	Dose	Rats	Inj.	Ovaries
	mg. eq.	*cc.*			*mg.*
Sheep SAP26	125	—	12	—	81
Sheep SAP26	125	1.0	3	C	85
Sheep SAP26	125	1.0	3	S	14
Sheep SAP26	100	—	3	—	54
Sheep SAP26	100	1.0	6	C	91
Sheep SAP26	100	1.0	3	S	13
Hog 5HAP16	275	—	12	—	65
Hog 5HAP16	275	1.0	6	C	129
Hog 5HAP16	275	1.0	6	S	35
Hog 5HAP16	275	2.0	3	C	102
Hog 5HAP16	275	2.0	3	S	16
Horse EAP4640	6.5	—	6	—	77
Horse EAP4640	6.5	1.0	6	C	15
Horse EAP4640	6.5	1.0	6	S	10
Human AP3RKM7	3	—	3	—	40
Human AP3RKM7	3	1.0	3	C	10
Human AP3RKM7	3	1.0	3	S	11
Human AP3RKM7	4	—	5	—	58
Human AP3RKM7	4	1.0	3	C	13
Human AP3RKM7	4	1.0	3	S	11
Rat APA1	4	—	6	—	65
Rat APA1	4	1.0	6	C	13
Rat APA1	4	1.0	6	S	11
Cow BAP1401	500[1]	—	9	—	97
Cow BAP1401	500	1.0	6	C	23
Cow BAP1401	500	1.0	6	S	14
FSH404	500	—	15	—	69
FSH404	500	1.0	6	C	14
FSH404	500	1.0	6	S	11
PMS118JWN35	0.2 cc.	—	6	—	94
PMS118JWN35	0.2 cc.	1.0	6	C	10
PMS118JWN35	0.2 cc.	1.0	6	S	9
Chorionic gonadotropin PPU100	1 mg.	—	6	—	49
Chorionic gonadotropin PPU100	1 mg.	1.0	6	C	22
Chorionic gonadotropin PPU100	1 mg.	1.0	6	S	25

[1] 6 mg. of hemin was added to each 500 mg. equivalent of dry gland.

when combined. It was not possible, however, to give sufficient antisheep-pituitary rabbit serum to inhibit the action of 275 mg. of hog pituitary extract but 100 mg. combined with 4 cc. of the antiserum was inhibited (table 1).

There was only partial inhibition of the human chorionic gonadotropin when it was administered with antisheep-pituitary serum by either of the two methods. The explanation of the results is not ob-

vious at the present time, although it may be due to the limited aspecificity of the antisheep serum.

A noteworthy result of combined injection of small amounts or relatively weak antigonadotropic sera and relatively unpurified gonadotropins prepared from sheep, hog and cow pituitary glands is the augmentation of gonadotropins from these sources. Such effects have been mentioned earlier in this report and examples are found in tables 1, 2 and 3. These data suggest that in these sera two systems are acting: *a*) an augmentative one composed of protein (Evans, Simpson and Austin, 1933) and hemoglobin (Casida, 1936) if hemolysis of red cells occurs, and *b*) an inhibitory one consisting of an antigonadotropic substance. If there is insufficient quantity of the latter to inhibit completely the action of the gonadotropin, the augmentative system acts so as to increase the effectiveness of the remaining gonadotropin.

SUMMARY

Antigonadotropic sera developed in rabbits and goats are more effective in preventing the gonad-stimulating action of relatively unpurified extracts prepared from sheep, hog and cow pituitary glands when they and the gonadotropin are injected separately into immature rats, than when mixed *in vitro* before injection.

Inhibition of the gonad-stimulating action of extracts of sheep and hog pituitary glands was obtained by combined injections if a sufficient quantity of rabbit or goat antigonadotropic serum was given.

With the removal of much of the inert material from sheep pituitary extract it was found that combined injections of gonadotropin and antigonadotropic sera were as effective as when administered by separate injections.

Gonadotropins prepared by aqueous extraction of horse, human and rat pituitary glands, pregnant mares' serum, purified preparations of human pregnancy urine were prevented from acting by combined as well as by separate injections of serum. These results are explained on the basis of the greater gonadotropic activity of these preparations per unit of dry weight as compared to that of sheep, hog and cow pituitary glands.

REFERENCES

Casida, L. E.: *Proc. Soc. Exper. Biol. & Med.* 33: 570. 1936.
Evans, H. M., M. E. Simpson and P. R. Austin: *J. Exper. Med.* 58: 561. 1933.
Gurin, S., C. Bachman and D. W. Wilson: *J. Biol. Chem.* 128: 525. 1939.
McShan, W. H., and R. K. Meyer: *J. Biol. Chem.* 135: 473. 1940.
McShan, W. H., and R. K. Meyer: *Endocrinology* 28: 694. 1941.

ASSAY OF ADRENAL CORTICAL EXTRACTS IN ADRENALECTOMIZED RATS EXPOSED TO COLD[1]

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Most of the methods for determining the potency of adrenal cortical extracts are based upon the correction of physiologic changes which occur in the animal organism following adrenalectomy. The observations that adrenalectomized animals exhibit profound changes in electrolyte metabolism and survive only for a short time after operation (Pfiffner, Swingle and Vars 1934), show a markedly impaired muscle efficiency (Ingle 1936) and hepatic glyconeogenesis (Reinecke and Kendall 1942) have been used as the bases for various methods of assay.

The sensitivity of adrenalectomized animals to cold environments noted by Elliot (1914), Cannon and Querido (1924) and Wyman and tum Suden (1929) was shown by Hartman, Brownell and Crosby (1931) and by Widström (1935) to be decreased by the injection of adrenal cortical extracts. Selye and Schénker (1938) have made use of this phenomenon in the development of an assay method for the detection of minute quantities of adrenal cortical activity. This method has been used for the detection of active material in urine extracts (Weil and Browne, 1939) and in extracts of the adrenal cortex (Kendall, 1940). The practical importance of a method for the detection of such small quantities of cortical material suggested the present study which is concerned with certain factors involved in this method of assay.

METHODS

Approximately 300 female rats, 21 to 26 days of age, the weight of which varied from 46 to 61 gm. were used. They had been fed Purina fox chow and kept in quarters the temperature of which was maintained near 25° C. Bilateral adrenalectomy was performed by the usual dorsal approach, and the experiments were made 12 to 24 hours later.

For exposure to cold the animals were placed in a refrigerator at $1° \pm 1°$ C. with a relative humidity of 70 to 80 per cent. Each animal occupied an in-

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dividual compartment in a cage of 1/2 inch mesh wire and not more than 14 rats were exposed at the same time. The period of exposure was interrupted at hourly intervals for the measurements of the colonic temperature, the readings being taken with a sensitive mercury thermometer in a room at 8° C. The instrument was inserted into the colon 3.5 cm. beyond the anal opening, without struggling of the animal, and readings were taken within 1/2 min. The duration of exposure to low temperature was usually 4 hours; a few groups were exposed for a shorter period, and several for 5 hours. In each experiment one-half of the animals received injections of adrenal corti-

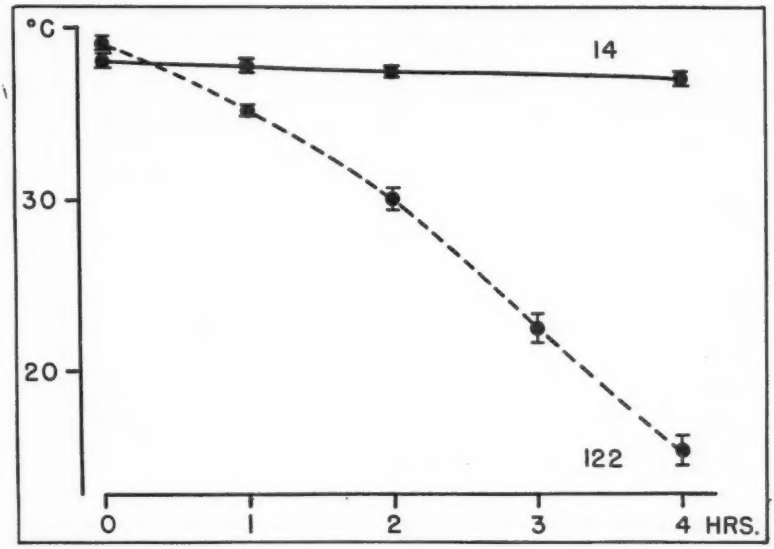

FIG. 1. Average colonic temperature of 122 adrenalectomized uninjected rats (19 groups), exposed for 4 hours, as compared with that of 14 unoperated controls (1 group). The vertical lines indicate the standard deviation.

cal extract, the other half serving as controls. The marked variations in the temperature curves for the control groups (fig. 2 and 3) has made the use of this technique necessary.

From 0.05 to 0.4 cc. of two commercial extracts, referred to as A and B, were administered subcutaneously 0 to 3 hours before exposure. In some instances the extracts were injected during exposure to cold.

The average colonic temperature of all animals which had been under the same experimental conditions was computed, as was the standard error. For two groups which had been under identical conditions except for the injected material, the standard error of the difference and the value of P were computed. As usual a value of P equal to or less than 0.05 was assumed to indicate a significant influence of the extract upon the average temperature.

RESULTS

A curve composed of the average colonic temperatures of 122 adrenalectomized animals (fig. 1) showed an initial temperature, 16 to 24 hours after operation, of 38.9±0.2°C. This temperature was 0.5° higher than that of normal rats or of sham-operated animals. After 4 hours of exposure the average temperature was 15°±0.8° C. and most of the animals had succumbed, whereas normal rats were able to maintain their initial temperature under these conditions for more than 6 hours.

Extract A. Amounts of extract A of 0.05, 0.1 and 0.2 cc. administered immediately before exposure had no significant influence upon

TABLE 1. COLONIC TEMPERATURES IN ADRENALECTOMIZED RATS AT 1° C. AND INJECTED WITH ADRENOCORTICAL EXTRACTS IMMEDIATELY BEFORE EXPOSURE TO COLD

Injected		Controls		t_1-t_2	(σ diff)[2] after 4 Hours	P after 4 Hours	Extract	Volume
No. of animals	t_1*	No. of animals	t_2†					
								cc.
13	23.9	14	19.8	4.1	13.0	<0.3	A	0.2
20	19.7	19	10.6	9.1	7.5	<0.01	A	0.3
18	21.9	17	12.7	9.2	4.2	<0.01	A	0.4
20	28.9	19	21.3	7.6	9.3	<0.02	B	0.05
21[1]	22.2	21	12.3	9.9	6.4	<0.01	A	0.4
22[2]	29.3[3]	12	18.4[3]	10.9[3]	6.3[3]	<0.01[3]	A	0.4

[1] Injected 1 hour before exposure.
[2] Injected 3 hours before exposure.
[3] After 2 hours.
* t_1 average temperature after 4 hours.
† t_2 average temperature after 4 hours.

the body temperature. Three-tenths and 0.4 cc. were both effective. There was a significant difference between rats receiving 0.3 cc. and controls after a period of 3 hours of exposure; and between those receiving 0.4 cc. and the controls after a period of 2 hours.

Extract B. This extract was more potent since 0.05 cc. administered immediately before exposure had a significant effect which was apparent after 4 hours of exposure. Smaller doses of this material were ineffective. In none of the animals in these groups was the colonic temperature maintained at its normal level for as long as one hour. The results are tabulated in table 1 and plotted in figure 2.

When 0.4 cc. of extract A was injected 0, 1 and 3 hours before exposure of the animals the results suggested that the protecting action of the extract was enhanced by an increase in the time interval between injection and exposure (fig. 3). This impression was supported by the fact that 0.4 cc. of extract A was ineffective, when administered during exposure, at a time when the average colonic temperature had fallen to 30° C. and by the fact that 0.2 cc. of the same extract was effective when given 3 hours before exposure.

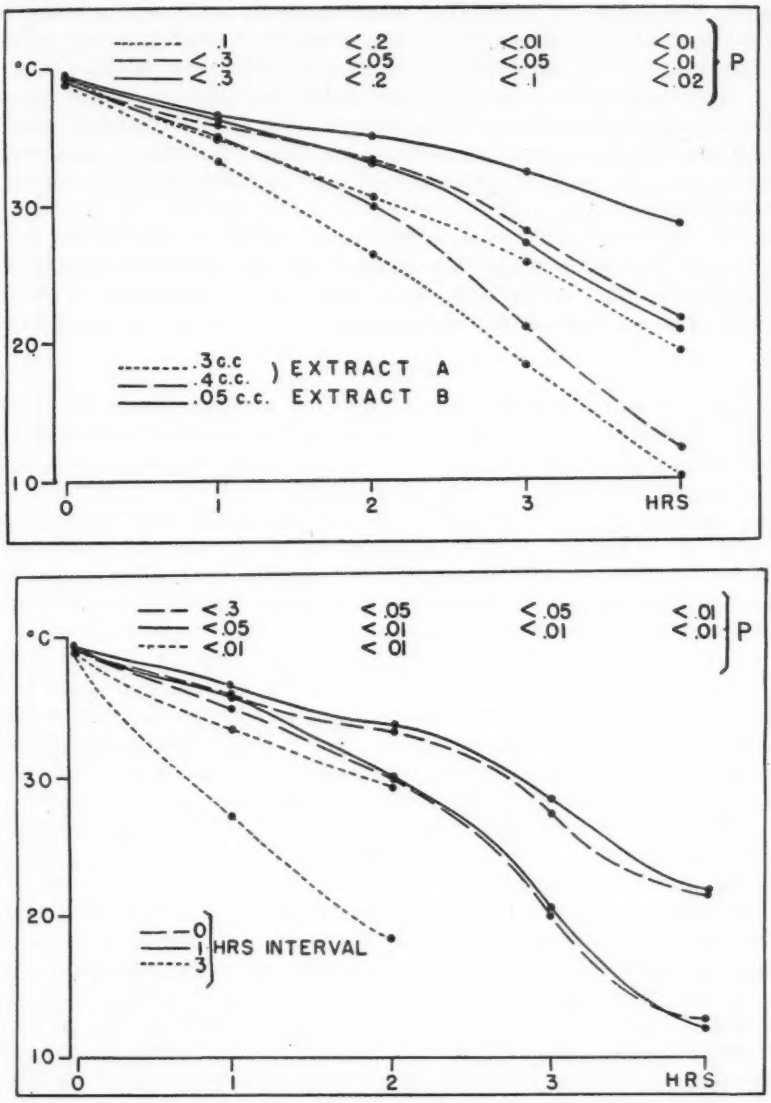

FIG. 2 (*upper*). Average colonic temperature of injected rats and controls. The extract was administered immediately before exposure. The lower of each pair of curves is the control.

FIG. 3 (*lower*). Average colonic temperature of injected rats and controls. Four-tenths cc. of extract A was injected at different intervals before exposure.

Body temperatures varied markedly within each group of animals, the degree of variability seeming to be somewhat greater in the injected animals than in the adrenalectomized controls, even though the latter not infrequently showed differences of 10° C.

The temperature curves for the uninjected animals, composed of the average readings for 2 or 3 groups totaling 12 to 21 animals which had been exposed on different days, also showed marked variations, which were as great or even greater than the differences between the temperature of the injected animals and the controls (fig. 2 and 3).

It was impossible to determine exactly when an animal had succumbed: the degree of spontaneous activity and response to mechanical stimulation decreased very gradually, and not infrequently seemingly dead animals recovered when put back in a warm atmosphere.

DISCUSSION

These findings confirm those of previous workers (Hartman, *et al.*, 1931; Widström, 1935) that adrenocortical extracts have a beneficial effect upon adrenalectomized rats exposed to low temperatures, and they agree with those of Selye and Schenker (1938) to the extent that the minimal effective amount (0.05 cc. of extract B) was of the same order of magnitude as that obtained by their method (0.03 cc.). These workers exposed 2 groups of 9 adrenalectomized rats each to low environmental temperatures. One group received the cortical material, divided into 3 subcutaneous doses: one immediately before, one after 3 hours and a third after 6 hours of exposure. The smallest total dose capable of keeping 6 animals alive, at the time when 6 of the controls had succumbed, was found to be 0.03 cc., and was called 1 unit. The test never lasted longer than 8 hours. The simplicity of their method and the relatively small number of animals used would make it useful as a method for assaying unknown preparations.

However, certain experimental findings and statistical considerations indicate that the following factors should be taken into account in the use of this method: *a*) The endpoint (death) is very difficult to determine, and it is virtually impossible to assign a definite time interval to the duration of life. *b*) Because of the ineffectiveness of large amounts of potent material when administered during exposure, at a time when the body temperature is falling, it is highly questionable whether the second and third injection given in the method of Selye and Schenker will have an effect upon body temperature or survival. *c*) Statistical considerations, as recently pointed out by Wilson (1941), taking into account the number of animals used and the criteria of effectiveness, show that a value of P considerably greater than 0.05 is obtained in the case of a 'positive' outcome of the test. This is true whichever of four mathematical methods applicable to this situation is being applied.

It should also be pointed out that in order to obtain significant

results, much larger numbers of animals are needed when death is used as an end point than in case of a quantitative index such as colonic temperature. Although a cold test, such as that described above, gives more reliable results, it too has practical disadvantages. Relatively large numbers of animals, all of the same age and weight, are necessary; temperatures have to be taken frequently; a dose-response reference curve for small amounts of extract cannot be constructed, and the minimal effective dose has to be determined each time.

SUMMARY

Experiments have been made on an assay method for adrenal cortical extracts involving the maintenance of the body temperature of adrenalectomized young rats exposed to an environmental temperature of 1° C. A single dose of 0.05 cc. of one commercial extract administered immediately before exposure decreased the rate of fall of the body temperature to a significant degree. A second commercial extract was one-sixth as active, 0.3 cc. being required for a comparable effect. Protection was afforded when 0.2 cc. was given 3 hours prior to exposure but no effect was observed when large amounts were injected after a fall in body temperature had occurred. Certain advantages and difficulties of this method of assay are discussed.

My thanks are due to Dr. E. B. Astwood for his advice and criticism.

REFERENCES

CANNON, W. B., AND A. QUERIDO: *Proc. Nat. Acad. Sciences* 10: 245. 1924.
ELLIOTT, T. R.: *J. Physiol.* 49: 38. 1914.
HARTMAN, F. A., K. A. BROWNELL AND A. A. CROSBY: *Am. J. Physiol.* 98: 674. 1931.
INGLE, D. J.: *Am. J. Physiol.* 116: 622. 1936.
KENDALL, E. C.: *Proc. Staff Meetings Mayo Clinic* 15: 297. 1940.
PFIFFNER, J. J., W. W. SWINGLE AND H. M. VARS: *J. Biol. Chem.* 104: 701. 1934.
REINECKE, R. M., AND E. C. KENDALL: *Endocrinology* 31: 573. 1942.
SELYE, H., AND V. SCHENKER: *Proc. Soc. Exper. Biol. & Med.* 39: 518. 1938.
WEIL, P., AND J. S. L. BROWNE: *Am. J. Physiol.* 126: 652. 1939.
WIDSTRÖM, G.: *Acta Med. Scand.* 87: 1. 1935.
WILSON, E. B.: *Science*, 93: 557. 1941.
WYMAN, L. C., AND C. TUM SUDEN: *Am. J. Physiol.* 89: 362. 1929.

STUDIES OF THE ZIMMERMANN REACTION: FACTORS AFFECTING THE COLOR INTENSITY; RELATION OF MOLECULAR STRUCTURE TO COLOR PRODUCTION[1,2]

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In the application of the Zimmermann method for the assay of 17-ketosteroids (Zimmermann, 1935; 1936) various modifications of the general procedure have come into use. These modifications involve variations in the concentrations of and the solvents for potassium hydroxide, the concentrations of m-di-nitrobenzene, duration and temperature of heating for the development of the color, and additional factors. Those who have used alcoholic potash have found that the calibration curves of androsterone and dehydroisoandrosterone are identical (Callow, *et al.*, 1938; Langstroth and Talbot, 1939; Talbot, *et al.*, 1940), but others, who have used aqueous potassium hydroxide, have found varying degrees of difference (Neustadt, 1938; Friedgood and Whidden, 1939; Holtorff and Koch, 1940; Saier, *et al.*, 1941). These differences in procedure and in assay values, together with our own experience with the chromogenic effects obtained with different hormones and extracts of biological material, prompted us to study the effect of a number of factors which affect the chromogenic value of androsterone and dehydroisoandrosterone, and in addition to assay a number of hormones and other substances for their chromogenic value. The following factors were studied: *a*) the time of heating in the water bath at 25° C. *b*) the concentration of m-dinitrobenzene; *c*) the concentration of potassium hydroxide; *d*) the chromogenic value of a number of substances in relation to that of androsterone.

PROCEDURE

The Holtorff-Koch method (Holtorff and Koch, 1940) was adopted as a general procedure, except that absolute alcohol was used as the solvent for the substances to be tested and for m-di-nitrobenzene, and that variations were introduced for the time of heating in the water bath. The substances

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to be assayed were prepared in stock solutions in absolute alcohol at 25° C. They were generally diluted with absolute alcohol at 25° C., so that 0.01 cc. contained 5 micrograms of substance. Aliquots less than 0.2 cc. were made up to that volume with absolute alcohol. The m-di-nitrobenzene (Eastman Kodak Co. #99) was also dissolved in absolute alcohol and was kept in the dark at room temperature. The potassium hydroxide (Baker's C.P., in sticks) was dissolved in distilled water, filtered through a fine, glass sintered filter, and checked by titration. The temperature of the water bath was held at 25° C., controlled by thermostat to within 0.1° C. The determinations were made with an Evelyn photoelectric colorimeter. The absorption constant was calculated on the basis of Beer's Law by dividing (2−log.G) by the number of micrograms of the substance used. It was left in this form for comparison purposes, since the total volume was identical in all of the assays. All chromogenic values are expressed in terms of the absorption constant.

EXPERIMENTAL RESULTS

Effect of the time of heating in the bath water at 25° C. Aliquots of androsterone were used, containing 20, 40, 60, 80 and 100 μg. All aliquots were employed in duplicates. The heating time was selected for 30, 45, 60 and 75 minutes. Fairly constant color intensities were obtained for each of the heating periods for the concentration range indicated. However, the optimum heating time at 25° C. with 5N potassium hydroxide and 2 per cent m-di-nitrobenzene appears to be about 60 minutes, which gives a distinctly greater color intensity than shorter lengths of heating time, while longer heating does not appreciably increase the color intensity.

Effect of variation of the concentration of m-di-nitrobenzene. The 5 concentrations of m-di-nitrobenzene employed were 2.0, 1.6, 1.16, 0.75 and 0.30 per cent solutions in absolute alcohol. Aliquots of 20, 50 and 75 μg. of androsterone and dehydroisoandrosterone were determined in duplicates. With both of the steroids employed, the color intensity increased with increase in concentration of m-di-nitrobenzene, using 5.025N KOH and heating for 60 minutes at 25° C. Fairly constant values were obtained with any of the concentrations of m-di-nitrobenzene employed, but the lowest concentration gave the least constant results, while the highest concentration produced the greatest color intenstiy. A somewhat greater color intensity appears to be possible with the concentration of the reagent above 2 per cent, but the employment of such higher concentrations is contraindicated by at least three considerations, namely: *a*) the increase in color would be comparatively slight; *b*) an increase in the concentration also produces a higher color for the reagent blank, thus reducing the useful range for the assay of unknowns; *c*) partial precipitation of the m-di-nitrobenzene is likely if the concentration is increased. A heating period of 60 minutes at 25° C. and 2 per cent m-di-nitrobenzene were employed in all subsequent determinations.

Effect of variation of the concentration of potassium hydroxide.

The 8 concentrations of potassium hydroxide employed varied from 1.0 to 8.0N. Aliquots of 20, 50, 75 and 100 μg. of androsterone and dehydroisoandrosterone were assayed in duplicates. Readings were made with Rubicon filters ranging from 420 to 660. These numbers

TABLE 1. AVERAGE CHROMOGENIC VALUES OF ANDROSTERONE AND DEHYDROISOANDROSTERONE WITH KOH CONCENTRATIONS VARYING FROM 1.0 TO 8.0 N. RANGE OF RUBICON FILTERS, NOS. 420 TO 660

Rubicon Filter No.[1]	Substance A[2] and DA	Normality Concentration of KOH							
		1.000	2.01	2.96	4.02	5.037	6.021	7.018	7.96
		K.10[4*]							
420	A	12.00	25.70	35.80	42.90	44.90	42.10	46.60	
	DA	15.30	29.60	42.70	46.20	46.50	41.60	44.10	
	K_{DA}/K_A	1.27	1.15	1.19	1.08	1.04	0.99	0.95	
490	A	15.60	36.00	51.40	71.30	83.10	85.30	88.20	96.80
	DA	21.70	43.30	63.80	80.60	88.10	86.10	88.80	97.10
	K_{DA}/K_A	1.39	1.20	1.24	1.13	1.06	1.01	1.01	1.00
520	A	15.20	34.80	51.60	73.80	88.10	95.10	98.80	110.80
	DA	20.95	43.30	63.95	84.00	95.60	97.10	101.10	111.90
	K_{DA}/K_A	1.38	1.24	1.24	1.14	1.085	1.02	1.02	1.01
540	A	13.30	29.90	44.20	63.40	76.90	84.00	89.60	95.90
	DA	18.00	36.40	54.80	72.00	82.60	85.70	90.30	96.50
	K_{DA}/K_A	1.35	1.22	1.24	1.14	1.08	1.02	1.01	0.95
580	A	8.00	17.10	26.40	39.20	48.70	55.60	59.20	62.20
	DA	10.50	21.20	32.00	44.20	51.60	55.30	59.50	64.20
	K_{DA}/K_A	1.31	1.24	1.21	1.13	1.06	0.99	1.00	1.03
600	A	5.90	12.40	19.20	29.40	36.80	42.80	45.50	47.90
	DA	7.50	15.30	23.10	32.20	38.40	42.10	45.60	49.90
	K_{DA}/K_A	1.27	1.23	1.20	1.10	1.04	0.98	1.00	1.04
620	A	3.90	8.00	12.40	19.20	24.80	29.60	31.60	32.80
	DA	4.70	9.80	14.80	21.40	25.80	28.70	31.50	33.80
	K_{DA}/K_A	1.21	1.22	1.19	1.11	1.04	0.97	1.00	1.03
635	A	2.40	5.00	8.00	12.60	16.50	19.50	20.50	22.20
	DA	3.00	6.20	9.30	13.80	17.10	19.10	20.80	22.70
	K_{DA}/K_A	1.25	1.24	1.16	1.09	1.04	0.98	1.01	1.02
660	A		2.50	3.60	6.40	7.70	9.00	9.30	11.70
	DA		3.00	4.30	6.80	8.00	8.60	9.80	11.50
	K_{DA}/K_A		1.20	1.19	1.06	1.04	0.96	1.05	0.98

[1] The filter numbers specify the approximate mean wave length of the transmitted band.

[2] A = androsterone; DA = dehydroisoandrosterone.

[4*] K = Absorption Factor = $(2 - \log. G)/\mu g.$

specify the approximate mean wave length of the transmitted band. Table 1 shows the average absorption values for these steroids for the conditions indicated above. In addition, the relative chromogenic values of androsterone and dehydroisoandrosterone are given for each set of determinations.

Chromogenic value of various hormones and other substances and

TABLE 2. ABSORPTION CONSTANTS[1] OF VARIOUS STEROIDS AND OTHER SUBSTANCES AND THEIR RELATION TO ANDROSTERONE VALUES. RUBICON FILTERS, NO. 420 TO 660;[2] ABSORPTION FACTOR 10^4

Rubicon Filter No.	420	490	520	540	580	600	635	660
Androsterone (A)	53.30	86.90	91.40	79.00	49.20	37.90	16.50	7.60
Dehydroiso androsterone (DA)	55.20	92.00	99.00	85.00	52.10	39.60	17.10	7.90
K/K_A	1.04	1.06	1.084	1.08	1.06	1.045	1.04	1.03
Androstenediol	0.00	0.00	0.80	0.50	0.00	0.00	0.00	0.00
K/K_A	0.00	0.00	0.009	0.006	0.00	0.00	0.00	0.00
Testosterone (T)	42.90	38.30	40.00	36.00	24.60	21.40	14.70	9.40
K/K_A	0.81	0.44	0.44	0.46	0.50	0.57	0.89	1.24
Methyl testosterone (MT)	38.20	34.20	36.50	31.90	22.80	19.50	13.40	8.90
K/K_A	0.72	0.39	0.40	0.40	0.46	0.52	0.81	1.17
K/K_T	0.89	0.89	0.89	0.89	0.92	0.91	0 .91	0.94
Progesterone	49.60	47.20	47.80	41.10	26.10	22.00	14.50	9.70
K/K_A	0.93	0.54	0.52	0.52	0.53	0.58	0.88	1.28
Pregnenolone	10.60	13.60	12.90	9.20	4.10	3.20	1.10	0.00
K/K_A	0.20	0.16	0.14	0.12	0.08	0.08	0.07	0.00
Desoxycorticosterone acetate	32.40	27.20	28.50	24.60	17.40	14.90	10.30	6.70
K/K_A	0.61	0.31	0.31	0.31	0.35	0.39	0.62	0.88
Estrone	53.40	87.70	94.30	80.70	50.60	38.30	17.80	8.70
K/K_A	1.00	1.01	1.03	1.02	1.03	1.01	1.08	1.15
α-Extradiol	4.60	3.50	3.80	2.80	1.10	0.70	0.20	0.00
K/K_A	0.09	0.04	0.04	0.04	0.2	0.02	0.01	0.00
α-Estradiol dipropionate	1.20	1.10	1.20	0.60	0.00	0.00	0.00	0.00
K/K_A	0.02	0.01	0.01	0.008	0.00	0.00	0.00	0.00
Estriol	2.70	2.40	2.60	1.90	0.60	0.30	0.16	0.00
K/K_A	0.05	0.03	0.03	0.02	0.01	0.008	0.01	0.00
Sodium dehydrocholate	13.90	8.30	7.70	6.50	4.40	3.70	2.40	1.70
K/K_A	0.26	0.10	0.08	0.08	0.09	0.10	0.15	0.22
Sodium deoxycholate	0.00	0.00	0.30	0.20	0.00	0.00	0.00	0.00
K/K_A	0.00	0.00	0.003	0.003	0.00	0.00	0.00	0.00
Acetone	101.00	63.10	63.50	53.60	37.20	31.00	21.50	15.90
K/K_A	1.89	0.73	0.70	0.68	0.76	0.82	1.30	2.09

[1] The absorption constants are average values for all concentrations used, as given in the text.

[2] The filter numbers specify the approximate mean wave length of the transmitted band.

their relative value to androsterone. In addition to androsterone and dehydroisoandrosterone the following substances were assayed, each concentration in duplicate: androstenediol (50, 75 and 100 μg.), testosterone (30, 50 and 100 μg.), methyl testosterone (30, 50, 75 and

100 μg.), progesterone (40, 80 and 120 μg.), pregnenolone (50, 75 and 100 μg.), desoxycorticosterone acetate (30, 50, 100 and 150 μg.), estrone (40, 80 and 120 μg.), α-estradiol (40, 80 and 120 μg.), α-estradiol dipropionate (40, 80 and 120 μg.), estriol (40, 80 and 120 μg.), sodium dehydrocholate (500 μg.), sodium desoxycholate (500 μg.) and acetone (100 and 200 μg.). The average absorption value of 4 aliquots for 15,

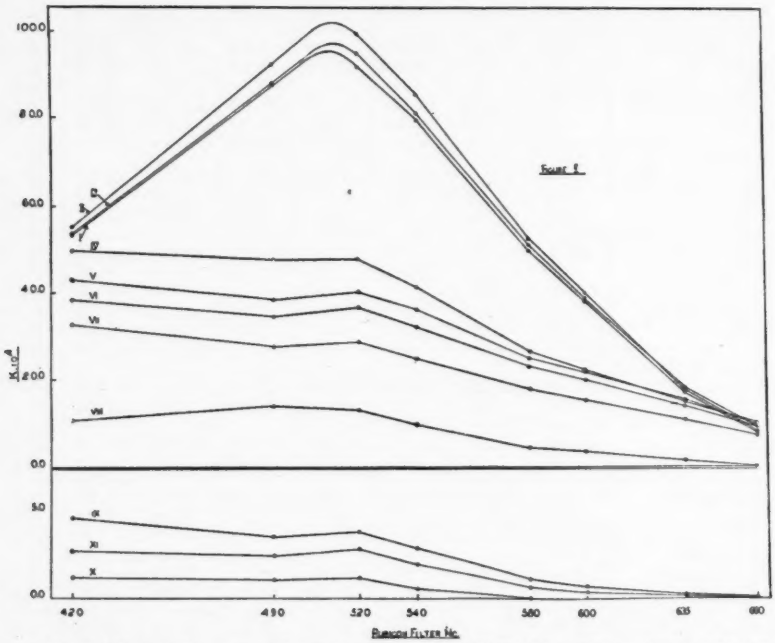

FIG. 1. Absorption curves for various steroids with Rubicon filters 420 to 660. The numbers specify the approximate mean wave length in millimicrons of the transmitted band. Curves: *I*, androsterone, *II*, dehydroisoandrosterone, *III*, estrone, *IV*, progesterone, *V*, testosterone, *VI*, methyl testosterone, *VII*, desoxycorticosterone acetate, *VIII*, pregnenolone, *IX*, α-estradiol, *X*, α-estradiol dipropionate, *XI*, estriol. Absorption constants are the average values for the concentrations as given in the text. Heating time 60 minutes at 25° C. Two per cent m-di-nitrobenzene. Potassium hydroxide 5.050N.

25, 50, 75 and 100 μg. of androsterone was used as a standard for comparison. The color was developed with 5.050N KOH. Readings were made with filters 420 to 660. Table 2 gives the average absorption constants of these substances for each of the filters employed, the relative value in comparison with androsterone, and in the case of methyl testosterone the ratios of its absorption constants to those of testosterone. Figure 1 gives the absorption curves for the various steroids and other substances employed with the above filters.

DISCUSSION

Fairly constant absorption constants were obtained with both androsterone and dehydroisoandrosterone with any of the concentrations of potassium hydroxide employed. With increasing concentrations of potassium hydroxide the color of the reagent blank becomes more intense, especially above 6.0N, so that the use of KOH above 6.0N is impractical on that account alone. The use of 5.0 to 6.0N KOH thus appears to establish optimum conditions for greater sensitivity. At the expense of considerable sensitivity a lower concentration of KOH might be useful to differentiate between androsterone and dehydroisoandrosterone.

Of the steroids under consideration only dehydroisoandrosterone and estrone approximate and exceed the absorption value of androsterone. With all of the other steroids the values are much lower, but they are negligible only for androstenediol, α-estradiol, α-estradiol dipropionate and estriol. The absorption curves (fig. 1) for the three 17-ketosteroids run approximately parallel. The curves of all of the remaining steroids are relatively flat and without any decided absorption peak for wavelengths 420 to 520 millimicrons. From 520 $\mu\mu$ on the curves of all of the steroids show a continuous drop to very low or zero values at 660 $\mu\mu$. Testosterone, methyl testosterone, progesterone and desoxycorticosterone acetate, which differ only in the radicles attached to C_{17}, show similar absorption curves, the highest for progesterone with a carbonyl in the side chain, somewhat lower and parallel values for testosterone and methyl testosterone, and lowest for desoxycorticosterone acetate. The influence of the methyl group in place of the hydrogen on C_{17} may be observed by comparing testosterone and methyl testosterone. The effect of the ketone group on C_3 may be seen by comparing progesterone and pregnenolone. The effect of the absence of ketone groups is noted in androstenediol, α-estradiol and estriol. The influence of esterifying groups on the nucleus may be noted by comparing α-estradiol and α-estradiol dipropionate, and the effect of introducing a hydroxyl group is suggested by the values for α-estradiol and estriol.

Certain of the bile acids produce a color which may effect the absorption value in 17-ketosteroid Zimmermann procedures, thus 3,7, 12-tri-keto cholanic acid ("Decholin") produces a color with an absorption value equivalent to 8 per cent of the androsterone effect, but the color produced with desoxycholate, which is free from carbonyl groups in the nucleus, is negligible. Even such a substance as acetone produces considerable color, 70 per cent of the androsterone value on a weight comparison basis. However, the color produced is qualitatively very different and quantitatively does not appear to follow Beer's Law.

Of the various sterols here studied figure 1 shows diagrammati-

cally the curves (I to VIII) for substances which are quantitatively important in the Zimmermann type of assay, while the remaining curves (IX to XI) indicate substances which may be neglected.

SUMMARY

A study has been presented showing how various factors affect the color production when m-di-nitrobenzene reacts with certain ketosteroids and other substances, using aqueous KOH as one of the reagents.

The effect on the color produced with androsterone and dehydroisoandrosterone by variations in the time of heating at 25° C., by employing different concentrations of m-di-nitrobenzene, and by using different concentrations of aqueous potassium hydroxide has been discussed.

The study of the color produced by 14 different steroids and by acetone under specified conditions has been presented with reference to *a*) the absorption constant for each of these substances, *b*) the value of these constants in terms of androsterone equivalents, *c*) the variations of the color as related to the molecular structure of these substances, and *d*) the general importance of the color produced from the quantitative standpoint in Zimmermann techniques for the assay of ketosteroids.

REFERENCES

CALLOW, N. H., R. K. CALLOW AND C. W. EMMENS: *Biochem. J.* 32: 1312. 1938.
FRIEDGOOD, H. B., AND H. L. WHIDDEN: *Endocrinology* 25: 919. 1939.
HOLTORFF, A. F., AND F. C. KOCH: *J. Biol. Chem.* 135: 377. 1940.
LANGSTROTH, G. O., AND N. B. TALBOT: *J. Biol. Chem.* 129: 759. 1939.
NEUSTADT, R.: *Endocrinology* 23: 711. 1938.
SAIER, E., M. WARGA AND R. C. GRAUER: *J. Biol. Chem.* 137: 317. 1941.
TALBOT, N. B., A. M. BUTLER AND E. MACLACHLAN: *J. Biol. Chem.* 132: 595. 1940.
ZIMMERMANN, W.: *Z. Physiol. Chem.* 233: 257. 1935.
ZIMMERMANN, W.: *Z. Physiol. Chem.* 245. 1936.

COLOR MEASUREMENT OF THE SEXUAL SKIN OF THE MACACUS RHESUS BY THE MUNSELL SYSTEM OF COLOR ANNOTATION[1]

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DURING a preliminary period of observation to determine the characteristics of the menstrual cycle of each of a group of recently purchased Macacus rhesus monkeys, the changes in the color of the sexual skin from day to day and from early morning through the daylight hours seemed noteworthy. Subsequent attempts to correlate such terms as orange-red, blue-purple, pale and intense with the phases of the menstrual cycle were unsatisfactory. The scale of 10 different colors as described by Corner (1932) was not accessible and it was difficult for novices in the field to establish reliable standards which would serve for comparison with that described by Corner.

The problem of a thorough study of color changes in the sexual skin of the female macaque appeared to be one requiring the skill of an artist in the mixing and matching of colors, and of an analyst to describe them. Such was the approach made to the problem, in the process of which a method of exact color analysis was found, the system of Munsell (1929). The swatches of unknown colors were matched to the known standards and the color notations were recorded. Color, under such circumstances becomes another tool for investigation of biological phenomena.

COLOR ANALYSIS

According to Munsell[3] color has three elements for measurement as definite as the three dimensions of a box, any one of which may be determined independently of the other two. A color with a Munsell notation may be reproduced exactly and the system has found wide application commercially. Special colors, the elements of which may not match the master standards can be analyzed with a color motor, the results substituted in a formula which is solved and the elements expressed in decimals of the standard term.

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[1] Aided by a grant for research from the American Medical Association.

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[3] Since the death of A. H. Munsell in 1918 the Munsell Color Company, Inc., has been responsible for extension of the work in color analysis. The Munsell Book of Color containing hand tinted standards may be found in libraries.

Swatches of the standard annotated colors may be purchased for a nominal sum[4] and a small handbook describing the system has been prepared by Cooper (1929).

The three elements of color are *a*) hue, or the tint, such as red, yellow or green; *b*) value, which may be dark, medium or light; and *c*) chroma, or the intensity of the color.

Hue. Hue, the first characteristic of a color that the eye detects, refers to the colors of the spectrum beginning with red (fig. 1), pro-

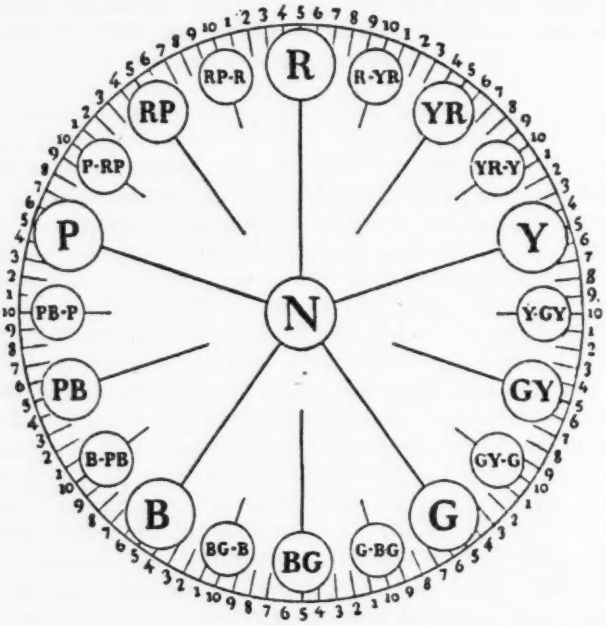

FIG. 1. Diagram showing the 5 principal hues, the 5 intermediate hues, the 10 secondary intermediate hues, and the 80 special intermediate hues (indicated by the numerals, 1, 2, 3, 4, and 6, 7, 8, 9), in the 100 hue circuit. (Reproduced by permission.)

ceeding clockwise and including primary and secondary intermediate hues. The five principal hues (red, R; yellow, Y; green, G; blue, B; and purple, P) and the five primary intermediate hues, (yellow-red, YR; green-yellow, GY; blue-green, BG; purple-blue, PB; and red-purple, RP), each have 10 numerical steps of transition from one to the other, and each lies opposite the fifth of the 10 steps. The secondary intermediate hues lie opposite the tenth transition step (red-yellow red, R-YR, is 10 R, or the last red hue before the first yellow-

[4] F. G. Cooper's book, "The Munsell Manual of Color" may be obtained from the Munsell Color Company, Inc., Baltimore, Md.; the swatches of color from the International Printer's Ink Corp., New York City.

red hue, 1YR). The hues are written as the initials of the colors preceded by the number of the hue transition, *i.e.* 1R, 2R, 3R, 4R, R (the 5 is omitted in writing) 6R, 7R, 8R, 9R; 10R is usually written in the term of the secondary intermediate hue which it represents, *i.e.* R-YR or red-yellow red.

Value. Value is the term for describing the lightness or darkness of a color. Reference to the color sphere in figure 2 shows that value

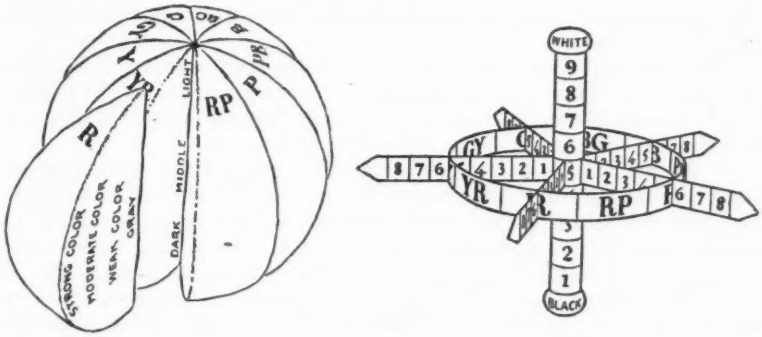

FIG. 2, (*left*). An orange assumed to represent the color sphere, with a segment displaced to show the neutral gray center, which is black at the bottom and blends upward through increasing lightness to white at the top. Note the change in color strength (chroma) outward from the center.

FIG. 3, (*right*). Diagram showing hue, value, and chroma in their relation to one another. The circular band represents the hues in their proper sequence. The upright center axis is the scale of value. The paths pointing outward from the center show the steps of chroma, increasing in strength as indicated by the numerals. (All of these diagrams are reproduced by permission of the Munsell Color Co., Inc., Baltimore, Maryland, from F. G. Cooper's Munsell Manual of Color).

is the axis of the sphere. The value of colors may range from blackest black to whitest white, and in figure 3 the numerical terms are shown ranging from 0 to 10.

Chroma. Chroma refers to the intensity or strength of the color. Reference to the color sphere shows that weak chromas lie near the axis of the sphere, and the stronger chromas more peripherally. In figure 3 the numerical steps of chroma are diagrammed.

Uniformly, then, what might be described as a watermelon pink color, could be so written in the Munsell system of annotation, that one would be able to reproduce it exactly, or to match it accurately, and the notation would show if the red were pure red (R), hue, a light or dark color (value) or whether it were weak or strong in chroma. If the hue were red, the value light and the chroma strong, the notation might read 7R 7/14, the value and chroma being expressed as a fraction, value/chroma, following the designation of the hue as an initial preceded by the hue transition number. Another and equally good

color for watermelon pink might be more purely red in hue, dark in value and strong in chroma, *i.e.* R 4/14.

MATERIAL AND METHODS

One castrated and four normal female macaques were used for observation. The animals were well accustomed to the daily routine procedure of having the 36-inch chain in the metal ring of the harness snaffled with a hook and drawn through a rigid wire loop at one end of a broomstick. Each had a characteristic way of leaving the cage, proceeding along the corridor to a dark room and jumping upon a table illuminated with two 500-watt daylight-blue incandescent bulbs.

The color matching was made at 11 o'clock each morning of the week. On Sundays, in the absence of the artist, the color swatches for the previous 3 days were used for comparison with the sexual skin for a less accurate color matching than was made on the other days of the week.

In the preliminary period of observation a distinct difference had been noted between the color at the root of the tail dorsal to the anus, and that of the sexual skin lateral to the ischial callosities. For this reason a color match for each of these areas was made for each animal each day. The colors for the two areas were mixed and matched on opposite ends of a strip of white water-color paper (Favor-Ruhl) 0.5×5 in., using pastels (Schmincke). With the exception noted for color matching on Sunday, the swatches for the preceding days were not used for comparison. Those for the first 10 days were discarded and the records include the colors for the 120 days between February 11 and June 10, 1939.

The swatches of color were analyzed by three graduate students in color work who worked independently, later checking their results for confirmation of accurate analysis. The room in which this work was done was equipped with a standard type of daylight illumination.

RESULTS

In the 5 macaques used in this study, under the existing environmental conditions, the hues ranged from red (R – 9R), through red-purple (RP-R – 4R) to purple (4RP – 9RP); the values from dark (3, 4, 5) to light (7, 8); the chromas from weak (3–7) to strong (11,12). The three elements of color as recorded for the castrated macaque in figures 4 and 5 can be re-translated into color by reference to the Munsell standards, just as the linear dimensions can be used in producing a box of stated size.

Colors in the sexual skin and tail base of the castrated macaque. An attempt was made to prepare a uterine fistula in this animal at the time of castration. Bleeding at the fistula was observed following cessation of estrogen administration but once in this animal which received 100 R.U. daily at the same hour on each of the 45 days that estrogen[5] was given. There was considerable swelling of the sexual

[5] Estradiol benzoate (Progynon-B) was supplied by Dr. E. Schwenk, Schering Corp., Bloomfield, New Jersey.

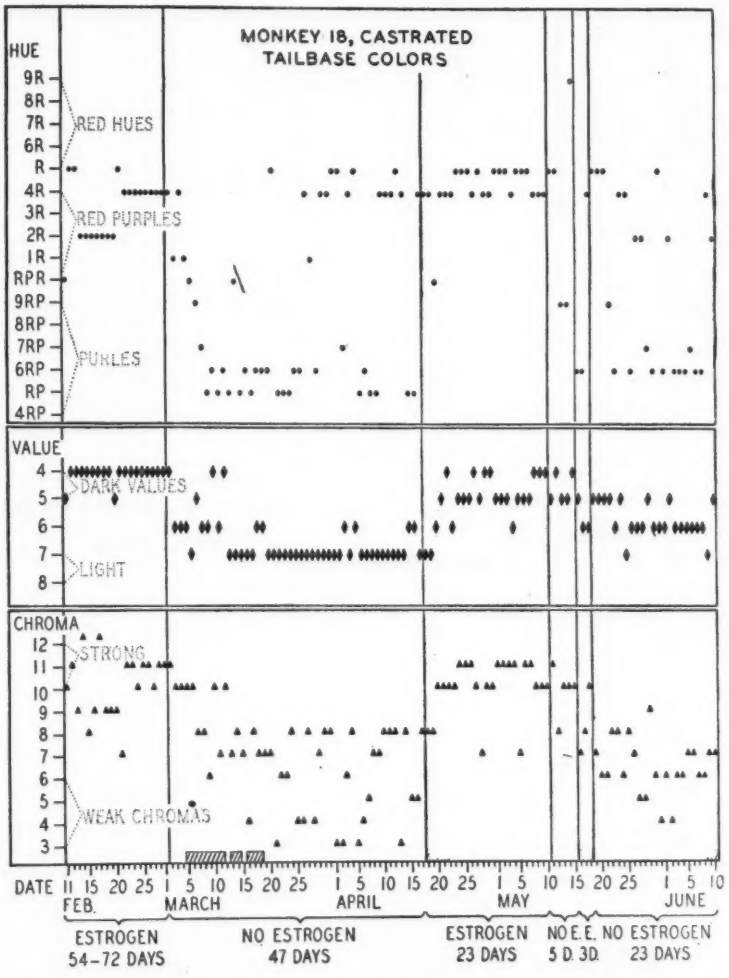

FIG. 4. Color record for tailbase area of a castrated female macaque for 120 days from Feb. 11 to June 11, 1939. Note that during the period of treatment with 100 R.U. of estradiol benzoate daily the hues are red and red-purple, the values dark and the chromas strong in contrast to the elements when no estrogen was given.

skin and corrugations or folding of the skin in the lumbosacral region, indicating that the animal was young.

From figure 4 it can be seen that the colors in the tailbase area were red-purple and red on 43 of the 45 days on which estrogen was given (95%), the values were dark (84%) and the chromas moderate and strong (100%). On 64 of the 75 days when estrogen was not given the hues in the tailbase area were purple and red-purple (85%), the values

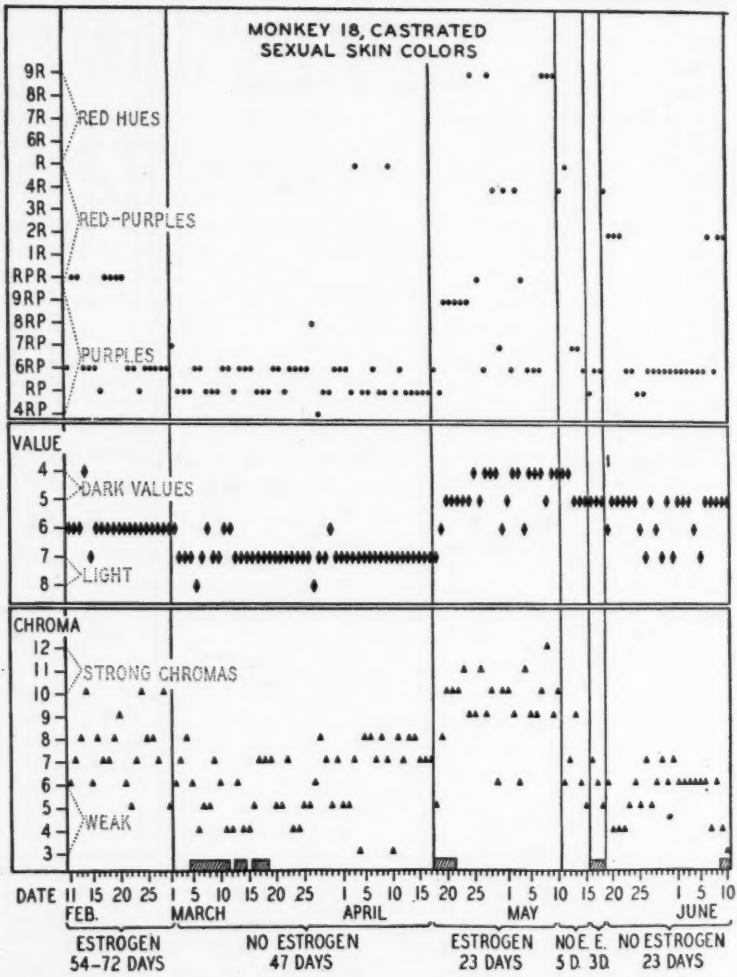

FIG. 5. Color record for the sexual skin area lateral to the ischial callosities of the same animal described in the legend for figure 4. Comparison of figures 4 and 5 shows that the tailbase colors during estrogen treatment were redder in hue, darker in value and stronger in chroma than the sexual skin colors. In the intervals without estrogen the colors of the two areas varied from day to day.

were light and middle (77%) and the chromas weak and moderate (85%). Briefly, under the influence of estrogen the tailbase colors in the castrated female macaque were redder in hue, darker in value and stronger in chroma than when the animal was not receiving estrogen.

The colors recorded for the tailbase area and the sexual skin varied from day to day during the intervals when estrogen was not adminis-

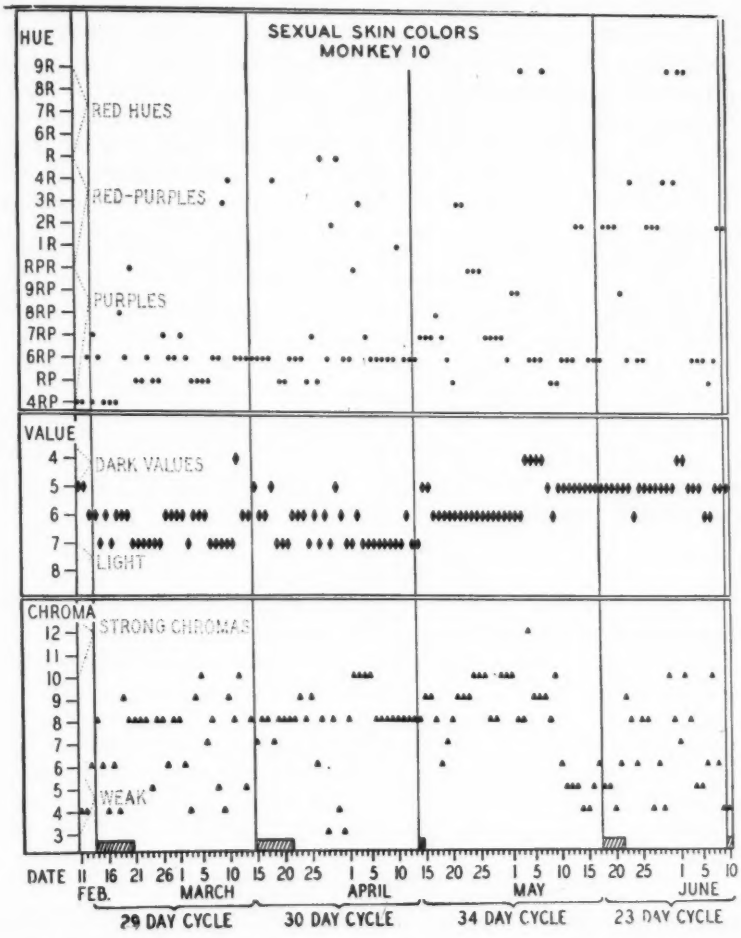

FIG. 6. Color record of a young intact female macaque through 4 cycles, sexual skin area lateral to the ischial callosities. There is little evidence for a color cycle, although the chromas tend to become weaker before the onset of menstruation. Note the wide variations in hue within the cycles, and that the hues were more often red and red-purple and the values dark during the period from April 10 to June 11 than from Feb. 11 to April 10.

tered (fig. 4, 5). During the period of treatment with estrogen the tailbase colors were more frequently red and red-purple (95%), the values dark (84%) and the chromas moderate and strong (100%) than was the case with the sexual skin colors, (35, 51 and 75%, respectively).

Intact macaques. Color data in graphic form are presented for the

sexual skin colors of but one of the 4 intact animals (monkey 10), since the series of animals was small. Monkey 10 was an adolescent as judged by the occurrence of periodic swelling. There was a trace of a color cycle referable to the element of chroma which often tended to become weaker on some days with the approach of menstruation (fig. 6). The values also shifted premenstrually, but this occurred throughout the cycle. The hues changed from purples and red-purples to reds and red-purples and back again with little reference to the occurrence of vaginal bleeding. During any 7-day period for each of the 4 animals, in either, and often in both, the sexual skin or tailbase area, there was a change in one or more of the 3 elements of color. These changes were of smaller degree and were less apparent during the last week in May and the 10 days in June.

Seasonal variations. The data recorded for the sexual skin and tailbase colors substantiated the observations that there is a seasonal variation in such colors. The colors recorded from April 11 to June 10 were more frequently red-purple and red in hue, and dark in value than they were from Feb. 11 to April 10. The strength of the chromas did not differ significantly in the two time-intervals.

DISCUSSION

The measurement of color in three terms as definite as the three dimensions of a box brings color into practical use for observations in the laboratory. The detailed method employed in the present study would not be necessary for routine observations as the commercially available swatches of the standard annotated colors are adequate. Some practice and experience in determining the values and chromas of the color swatches is necessary. A color blind person has less difficulty with this than the average because the third element, hue, is not seriously regarded, and full attention is given to the value and chroma.

SUMMARY

A method for recording color has been described and its usefulness demonstrated in the contrast of the colors recorded for the sexual skin and tailbase areas in a castrated macaque with and without estrogen treatment. The Munsell system of color annotation defines color in terms of hue, the name of the color, value as the amount of light in a color and chroma as the degree of strength in a color. These may be accurately determined by matching with color standards and recorded.

REFERENCES

Cooper, F. G.: Munsell Manual of Color. Waverly Press, Inc. Baltimore. 1929.
Corner, G. W.: *Anat. Rec.* 52: 410. 1932.
Munsell Book of Color: Munsell Color Company, Inc. Baltimore. 1929.

ALLOXAN DIABETES IN THE DOG[1]

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EXPERIMENTAL diabetes mellitus in dogs has been produced by pancreatectomy (Mehring and Minkowski, 1890; Allen, 1914) and by treatment with anterior pituitary extract (Houssay, Biasotti and Rietti, 1932; Evans, Meyer, Simpson and Reichert, 1932). The possibility that a third, or chemical, type of diabetes may be produced was first suggested by Dunn, Sheehan and McLetchie (1943). They found that alloxan has a specific necrosing action on both the pancreatic islet cells and the epithelium of the renal convoluted tubules when injected into rabbits intravenously or intraperitoneally in doses of 100 to 500 mg. per kg. Previously, Jacobs (1937) had observed that similar doses of alloxan produce in rabbits an initial hyperglycemia followed by severe and prolonged hypoglycemia of which most of the animals die unless glucose injections are given; Jacobs, however, did not report histologic studies. Dunn and co-workers confirmed the production of alloxan hypoglycemia and found that it is accompanied by a marked rise of blood urea nitrogen. Their remarkable discoveries suggested that alloxan may cause diabetes and that it or a related compound may play a rôle in the development of human diabetes, the more so as alloxan is a product of oxidative breakdown of uric acid. The authors were unable, however, to maintain animals alive for longer than two days after necrosis of the islet cells and did not succeed in producing chronic lesions by any form of prolonged treatment.

The purpose of our studies was to determine *a*) if there is a dosage of alloxan which would affect the pancreatic islet cells without damaging the renal parenchyma and *b*) if any species would survive such a dose long enough for metabolic studies.

We have confirmed the observations of Jacobs and Dunn and coworkers in the rabbit and, in addition, have extended our experiments to rats, guinea pigs, cats, dogs and pigeons, all of which were found to be sensitive in various degrees to alloxan. Permanent diabetes could be produced in rats, rabbits and dogs. The results of our experiments with dogs are most significant for our special problem and will be reported in this paper.

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TABLE 1. SUMMARY OF CLINICAL AND PATHOLOGIC FINDINGS IN 12 DOGS AFTER A SINGLE INJECTION OF ALLOXAN

Dog No.	Dosage Alloxan	Weight	Loss of Weight	Period of Survival	Clinical Course
	mg./kg.	*kg.*	*kg.*	*hr.*	
1	200	8.5		2	Sudden death with convulsions
206	150	10.4		1½	
214	125	10.0		8	
				days	
164	100	13.0	2.0	4	Diabetic uremic syndrome
203	100	16.2	2.8	7	
169	75	19.4	1.4	5	
219	75	12.4	2.0	18	Sustained diabetes
211	50	10.2	1.5	16	Sustained diabetes
719	50	14.2	2.1	16	Sustained diabetes
140	50	9.8	1.6	48	Sustained diabetes
108	50	12.0	0.5	indefinite	Transitory diabetes
600	25	10.5	0.5	indefinite	normal

EXPERIMENTAL PROCEDURES

Twelve dogs were injected intravenously with various amounts of alloxan. A 5 per cent solution in distilled water, freshly prepared, was used in each instance. The injection was given into the tibial vein. All dogs except one (dog 600) received only a single injection of alloxan. Dog 600 received a second injection two weeks after the first.

The animals were fasted for 14 hours prior to the injection. Every morning, 14 hours after the food had been removed from the cages, blood specimens were taken for chemical studies. Blood-sugar estimations were made daily, determinations of non-protein nitrogen (NPN), CO_2 and serum pH, total blood lipids, blood proteins and blood ketone bodies were made at intervals. The urine was examined both chemically and microscopically. Sugar excretion was determined on 24-hour specimens. As the total food intake was not estimated accurately no complete carbohydrate balances can be given.

Since we were interested in the period of survival of the animals and in the duration of the diabetic condition, no attempts were made at therapy. Insulin sensitivity tests, however, were performed.

In a few instances blood pressure determinations and temperature readings were made, and in one instance the respiratory quotient was determined.

For histologic studies thin slices of fresh tissues were fixed in Bouin's fluid

TABLE 1.—*Continued*

Dog No.	Biochemical Findings							Anatomical Findings
	NPN		FBS		Glucosuria	Urine Albumen	Ketone bodies	
	Average	Highest	Average	Highest				
	mg. %		*mg. %*					
1			77	80	0	0	0	
206		31	70	48 (lowest)	0	0	0	Normal
214		26	74	72	0	0	0	
164	189	275	359	800	4+	0	0	Pancreas: degranulation of beta cells
203	77	134	338	533	4+	2+	0	Kidney: necrosis of tubular epithelium
169	220	367	588	1049	4+	0	0	
219	32	44	240	291	4+	(+)	0	Pancreas: disappearance of beta cells
211	42	59	210	374	4+	(+) hematuria	0	Vacuolation of duct epithelium
719	24	34	245	297	4+	0	0	Kidney: normal except Dog 211 (see text)
140	32	35	250	389	4+	0	0	
108		22	102	274	transitory	0	0	Liver: fatty infiltration
600			88	96	0	0	0	Normal

and in absolute acetone. Cell types in the islets were stained with chrome hematoxylin-phloxin and with a modification of the Mallory-Heidenhain azan stain (Gomori, 1941). Other special stains used were the Bauer-Feulgen (1933) stain for glycogen, the Kóssa silver impregnation for insoluble calcium salts and the alkaline phosphatase (Gomori, 1939) stain. A number of sections were stained with hematoxylin and eosin. Frozen sections of livers were stained for fat with Sudan III.

RESULTS

It was found that 50 to 75 mg. of alloxan per kg. of body weight given intravenously in one injection produced in dogs a diabetic syndrome without symptoms of disturbed kidney function; such dogs survived for over 3 weeks.

The experimental findings are summarized in table 1 which demonstrates that the results can be divided into the following four main groups according to the dosage of alloxan as well as according to physiological and anatomical findings: *a*) death within 8 hours, *b*) hyperglycemia and glycosuria with severe renal damage, *c*) typical diabetes mellitus with slight or no renal pathology, and *d*) no effect due to subthreshold dose.

Group I. Doses of more than 100 mg. of alloxan per kg. given in a single injection intravenously to dogs not treated with alloxan previ-

ously,[2] were fatal within a few hours, as shown in dogs 1, 206 and 214 This is in agreement with the observations of Brunschwig and Allen (1943) who made their study simultaneously but independently. The dogs did not show any symptoms for the first hour but became weak, depressed and drowsy afterwards, until they died in sudden attacks of convulsions. The NPN remained normal during this period; the blood sugar fell to 48 mg. per cent in dog 206 but was unchanged in dogs 1 and 214. The blood pressure and temperature in dog 1 were normal until one-half hour before death. Microscopic examination of the organs failed to show any change. The cytology of the islets was normal. The cause of death in these dogs remains unexplained since it apparently was not due to hypoglycemia nor to kidney damage.

Group II. Seventy-five to 100 mg. of alloxan per kg., given intravenously in a single injection, produced a hyperglycemic-uremic syndrome to which the dogs succumbed within 4 to 7 days. They appeared well and normal during the first 24 or 48 hours after the injection but became sick, listless and drowsy later, which condition grew worse until they became semi-comatose. They took very little food or water from the second day on, losing weight rapidly, and were oliguric or anuric. They did not have convulsions at any time. The blood sugar showed a moderate initial hypoglycemia which was followed on the second day by an extreme hyperglycemia. The NPN rose steadily from day to day. The urine, when obtained, was found to contain sugar (4+ reduction) but albumen was present in one instance only, and ketone bodies were absent. Microscopically, no pathological elements were seen in the urine. Dogs 164, 169, and 203 belong in this group. The course of the first two is illustrated by figure 1.

Dog 164 weighed 13 kg. and was given a total of 1300 mg. of alloxan (100 mg. per kg). The initial blood sugar was 94 mg. per cent and it had fallen by the next morning to 42, at which time the NPN was 101 mg. per cent. On the third morning the blood sugar was 503 mg. per cent and the NPN 193 mg. per cent. At this time a biopsy from the pancreas was taken. The dog seemed to recover from the operation which was performed under sodium pentothal anesthesia. On the fourth morning, however, he was very weak, semi-comatose and anuric. The blood sugar was 800 mg. per cent, the NPN 275 mg. per cent. The blood pressure was normal, and the temperature was 101° F., rectally. The animal died on the fifth morning.

The biopsy specimen of the pancreas showed the islets to be much less conspicuous than normally and rather poorly delimited. Many of the islet cells were small with somewhat pyknotic nuclei and scanty cytoplasm (fig. 4). There were no granules in the beta cells. The alpha cells were well preserved and numerous. There was no vacuolation in either the islet or the duct epithelium. The autopsy specimen of the pancreas was not fresh enough for cytologic study. The kidney showed extensive necrosis of the convoluted tubules with calcification of many of them (fig. 5). There was a moderate amount of glycogen both in Henle's loops and in some of the convoluted tubules. The liver appeared normal grossly.

[2] It was found that dogs which have been treated with an ineffective first dose will show an increased resistance to alloxan.

Dog 169 weighed 19.4 kg. and was given a total of 1425 mg. of alloxan (75 mg. per kg.). Its initial blood sugar was 77 mg. per 100 cc. Twenty-four hours after injection the blood sugar was 67 mg. per cent and NPN 71.4 mg. per cent; on the third day the blood-sugar level reached 768 and rose on the fourth day to 1049 mg. per cent. The urine contained 9 gm. of glucose on that day. Weight loss was 1.2 kg. On the morning of the fifth day the animal was semi-comatose; the blood sugar was

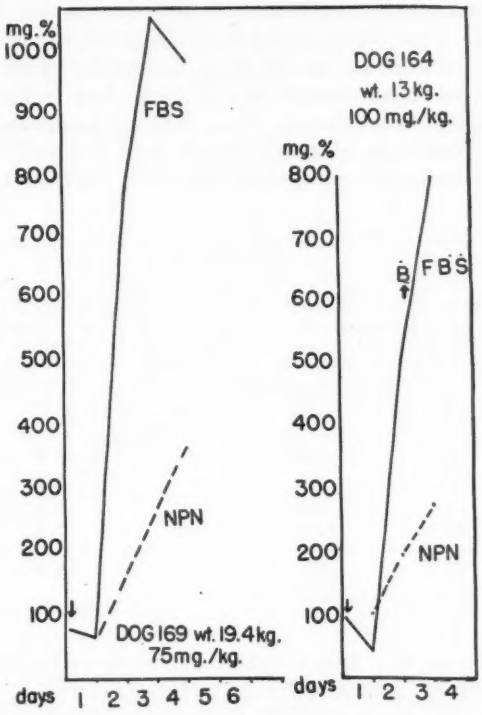

FIG. 1. Blood sugar (FBS) and blood urea nitrogen (NPN) levels in dogs 169 and 164 after 75 and 100 mg. per kg. of alloxan, respectively, in a single intravenous injection. ↓ indicates time of injection, ↑ B biopsy.

981 mg. per cent and NPN 367 mg. per cent. The urine contained neither albumen, blood nor casts but showed very strong (4+) reduction. The animal died on the next morning.

Histologically the islets appeared normal when stained with hematoxylin and eosin; however, the chrome hematoxylin stain revealed a complete absence of granules in the beta cells. The overwhelming majority of the islet cells were normal alpha, with a small number of non-granular cells of an unrecognizable type. There was no vacuolation of either islet or duct epithelium.

The findings in the kidney and the liver were identical with those in dog 164.

Group III. Dogs given 50 to 75 mg. of alloxan per kg. characteristically developed polydipsia and polyuria, hyperglycemia and glyco-

suria and lost weight. There was no evidence of disturbed renal function except in dog 211. These dogs appeared to be rather well throughout the first 2 weeks; they did not show any initial hypoglycemia but developed within the first days a hyperglycemia which, with the exception of dog 108, remained constant until the end of the experiment and ranged between 200 and 350 mg. per 100 cc. In dog 108 the hyperglycemic-glycosuric phase was transitory, lasting only 3 days, after which the sugar disappeared from the urine and the blood-sugar level returned to normal. In the case of dogs in which the diabetic condition became permanently established their behavior changed by the end of the second week. They became weak and sleepy, lost their appetite and were unsteady on their legs. The NPN remained within normal limits; it was noted, however, that the blood became

TABLE 2. BLOOD LIPIDS, CO_2 COMBINING POWER AND PH OF SERUM IN DOGS WITH ALLOXAN DIABETES

Dog No.	Total Plasma Lipids		CO_2 and pH in Serum		
	Days after injection	mg. per cent	Days after injection	CO_2 mM/L	pH
219	9	1063	10	11.9	7.29
	13	1980	14	12.8	7.31
211	9	663	10	19.2	7.33
	13	2980			
719	9	733	11	19.4	
	14	6300	16	17.8	7.31
140	10	617	20	16.2	
	19	5130			

heavily laden with fat. Quantitative determinations showed excessively high levels of fat (table 2). One of these dogs (211) had a transitory hematuria for about 4 days; otherwise in all other instances the urine was free of blood and casts and showed, at most, mere traces of albumen. It did not contain ketone bodies at any time. The concentration of sugar in the urine reached 7 per cent. The ketone bodies in the blood were found to be moderately elevated (6.8 mg. per 100 cc. in dog 219). The CO_2 combining power and the pH of the serum were slightly decreased. In dog 719 the respiratory quotient was determined on the seventeenth day of its diabetes and found to be 0.64 and 0.67 on two estimations.[3] Insulin sensitivity tests were performed in 3 dogs. The fasting animals were given 4 units of crystalline insulin subcutaneously and the hypoglycemic response was determined with blood sugar estimations 30, 60, 120 and 180 minutes after insulin injection. The marked insulin effect is represented in table 3.

[3] We are indebted to Dr. Irene Sandiford for this determination.

TABLE 3. INSULIN SENSITIVITY TESTS IN DOGS WITH ALLOXAN DIABETES

Dog No.	Days after Onset of Diabetes	Blood Sugar before and at Intervals after Subcutaneous Injection of 4 Units of a Solution of Insulin Zinc Crystals, mg.%				
		Fasting	30′	60′	120′	180′
211	13	243	223	102	61	66
140	23	320	270	250	120	91
600[1]	9	270	208	107	95	73

[1] After reinjection with an effective dose.

The course of events in the dogs in this group is illustrated in figure 2 which demonstrates the blood sugar and NPN levels in dogs 140 and 219.

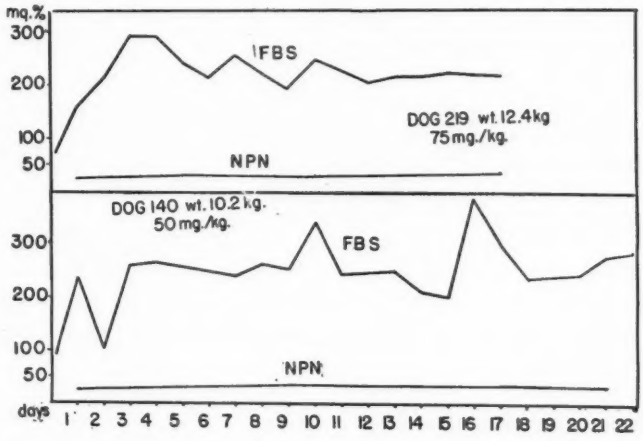

FIG. 2. Blood sugar (FBS) and blood urea nitrogen (NPN) levels in dogs 219 and 140 after 75 and 50 mg. per kg. of alloxan, respectively, in a single intravenous injection.

Dogs 140 and 219 were glycosuric from the second day after injection. They excreted occasionally as much as 28 gm. of sugar within 24 hours and on the average 10 to 20 gm. in 24 hours. They lost 1.6 kg. and 1.4 kg. of weight, respectively, within the first 10 days. In dog 219 the total blood lipids were 1063 and 1980 mg. per 100 cc. on the ninth and thirteenth diabetic day, respectively. On the fifteenth day the total blood proteins were 6.16 gm., the albumin/globulin ratio 0.89, CO_2 combining power of the serum 12.8 mM/L, and pH 7.31. On the seventeenth day the total acetone bodies in the blood were 6.86 mg. per 100 cc.

The blood lipids in dog 140 were 617 mg. per 100 cc. on the tenth and 5130 mg. on the nineteenth diabetic day.

On histologic examination in dog 219 the islets were rather small

and poorly delimited. Two cell types could be distinguished. Many were normal, well granulated alphas, and there were smaller agranular cells. There was a marked proliferation of small ducts, the lining of which was extremely vacuolated.

The kidney was normal histologically, except for small amounts of glycogen in Henle's loops and in some of the convolute tubules.

Grossly the liver showed a conspicuous mottling with irregular dark reddish-brown and dull yellowish areas. Microscopically there were irregular areas of extreme fatty infiltration. Often small groups of ballooned cells were found in the midst of fairly normal looking areas of tissue. All vacuoles were stained with Sudan III. At the periphery of the fatty areas many cells had pyknotic or fragmented nuclei and a homogeneous, eosinophil cytoplasm. Leukocytes were numerous in the sinusoids. Glycogen was found in the leukocytes only. With the phosphatase stain all liver cells, both fatty and normal, were stained very dark; the bile capillaries and sinus walls were outlined in black (fig. 7). This phosphatase reaction was far more intense and extensive than that observed in more than 30 control animals (fig. 9).

In dog 211 the islets were normal in size and outlines; they consisted mainly of normal alpha cells, with a small number of non-granular cells. No normal beta cells were visible. There was no vacuolation of either the islet cells or of the duct epithelium.

The kidney showed extensive necrobiosis involving mainly the straight segments of the distal convoluted tubules. Some of the tubules contained dark brown droplets in the epithelial lining (hemoglobin?); in some places the necrosed epithelial cells showed early calcification. There was some glycogen in Henle's loops.

Grossly and microscopically the liver was very similar to that of dog 219 in all respects except that *a*) there was a marked congestion, and *b*) degenerative changes consisting of nuclear fragmentation and pyknosis, and homogenization of the cytoplasm over large areas was conspicuous.

In dog 719 the biopsy specimen of the pancreas, taken on the third day, looked normal with the routine hematoxylin-eosin stain. However, special stains showed that the islets consisted of normal alpha cells and non-granular cells only. No normal beta cells were present. In the autopsy specimen of the pancreas the islets were rather small and scarce; they consisted of large numbers of well-preserved alpha cells with a few smaller non-granular cells (fig. 11). The duct epithelium was markedly vacuolated (fig. 10). The kidney was completely normal, except for some glycogen in Henle's loops.

Grossly the liver seemed to be markedly enlarged; its color was a dull yellowish-brown. Microscopically it showed marked fatty infiltration of a rather regular central and mid-zonal type (fig. 9). Only the most peripheral portions of the lobules were spared. The nuclei were well preserved throughout. There were a considerable number of

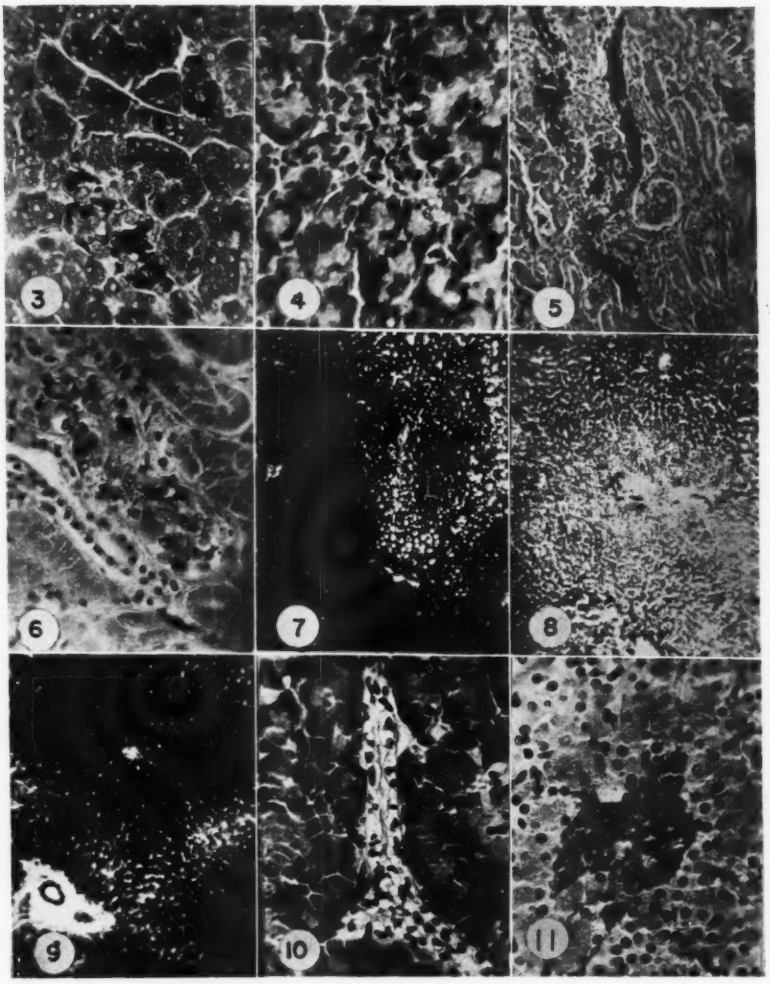

FIG. 3. Pancreas, dog 206. Chrome hematoxylin-phloxin stain. ×280. Normal islets with many dark staining beta cells.

FIG. 4. Pancreas, dog 164. Hematoxylin-eosin stain. ×280. An islet with many shrunken cells.

FIG. 5. Kidney, dog 164. Kóssa silver stain. ×85. Necrosis and calcification of renal tubules.

FIG. 6. Kidney, dog 169. Bauer-Feulgen stain. ×280. Droplets of glycogen in Henle's loops.

FIG. 7. Liver, dog 219. ×60. Phosphatase picture of the liver.

FIG. 8. Liver, ×60. Exceptionally strong phosphatase reaction in the liver of a control animal.

FIG. 9. Liver, dog 719. Fat stain of the liver. ×60.

FIG. 10. Pancreas, dog 719. Hematoxylin-eosin stain. ×280. Vacuolation of pancreatic duct epithelium.

FIG. 11. Pancreas, dog 719. Modified azan stain. ×280. An islet consisting almost exclusively of heavily granulated, dark alpha cells.

leukocytes in the sinusoids. The vacuoles all stained with Sudan III. This liver contained 31 per cent lipids, as determined chemically. Glycogen was present in the leukocytes and in fine droplets in scattered liver cells around the periphery of the lobules. The phosphatase picture was similar to that in the previous 2 cases.

In dog 108, the biopsy specimen was taken the ninth day after injection, two days after the blood-sugar level had returned to normal. With a hematoxylin-eosin stain the islets looked normal; with specific stains a small number of the cells were agranular; the granulated cells were all of the alpha type.

Group IV. Doses of 25 mg. per kg. of alloxan seemed to be ineffective. The sugar content of the blood and urine remained normal in dog 600 after such an injection. A biopsy from the pancreas, taken on the eighth day, showed completely normal islet cells. This dog later was given a larger dose and has since become diabetic.

COMMENT

Alloxan diabetes in dogs is characterized by *a*) prompt onset of hyperglycemia and glycosuria within 24 to 48 hours, sometimes preceded by a short hypoglycemic reaction if doses of 75 to 100 mg. per kg. are given; *b*) hyperlipemia and fatty degeneration of the liver which develop with 10 to 12 days. It differs from anterior pituitary diabetes in that *a*) a single injection suffices to produce permanent diabetes, and *b*) the animals remain sensitive to insulin.

The extremely high blood-sugar levels observed after doses of 75 to 100 mg. per kg. suggest that alloxan exerts its diabetogenetic action not only through necrosis of the insulin-producing islet cells but also by direct effect on the liver parenchyma. In totally depancreatized dogs the blood sugar seldom reaches such high values, at least never within the first few days. The same consideration may apply to dogs receiving 50 to 75 mg. per kg. and responding with a hyperglycemia and glycosuria comparable to that seen in other types of experimental diabetes. Here it could be questioned whether hyperlipemia and degenerative changes of the liver are secondary to lack of islet function or caused by the direct action of alloxan on the liver. In this regard it is noteworthy that no hyperlipemia was found in the one case of transitory diabetes (dog 108), and that Brunschwig and Allen (1943) found normal livers in their dogs with transitory diabetes which had received larger amounts of alloxan in split doses.

Histologically, the early changes in the islets were complete degranulation of the beta cells with shrinkage of the entire cell body. The number of the agranular cells gradually decreased, and by the end of the second week very few of them could be seen. Hydropic degeneration of the beta cells, so prominent in both surgical and pituitary diabetes, was never observed in this series of animals nor

were any mitoses found in islet cells. The alpha cells were unaffected; in fact, they seemed to increase in numbers. Although apparently the islets themselves became smaller and less numerous in the course of alloxan diabetes, no definite statement can be made to this effect because an actual decrease in total islet cell volume could be proven only by exact measurements made on many representative sections.

The most conspicuous feature of the pancreatic lesions in alloxan diabetes was the extreme vacuolation of the epithelium of all intralobular ducts in the later stages of the disease. This phenomenon has been observed, although in a much milder form, both in surgical (Allen, 1922) and in anterior pituitary (Richardson and Young, 1938) diabetes.

The extreme fatty infiltration of the liver, associated with degenerative and inflammatory changes, seems to be a specific effect of alloxan poisoning. Fatty infiltration without conspicuous inflammatory and degenerative changes is observed regularly in depancreatized dogs and occasionally in dogs with pituitary diabetes. Ham and Haist (1941) found fatty changes of the liver in only two animals, both of which showed degenerative changes in the alpha cells. In view of these findings Dragstedt (1943) suggested a possible rôle of alpha cells in the prevention of fatty liver. In our observations the alpha cells were invariably normal and present in large numbers in all animals. Further experiments will show whether this type of liver damage is preventable or curable as is the fatty liver of pancreatectomy.

The significance of the marked increase in phosphatase reaction far beyond the limits of the normal picture is not clear.

Higher doses of alloxan lead to severe lesions of the kidney, very similar to those caused by mercury. With smaller doses the kidney escapes damage. The infiltration of Henle's loops with glycogen is obviously due to the prolonged glycosuria.

The adrenals did not show appreciable changes in any of the animals. The cortex was of normal structure, and the medulla showed excellent chromaffin granulation.

SUMMARY

Intravenous administration of alloxan to dogs in a single dose exceeding 100 mg. per kg. caused death of the animals within a few hours. A dose between 75 and 100 mg. per kg. produced a diabetic-uremic syndrome of which the animals died within one week. A dose of 50 to 75 mg. per kg. produced typical diabetes without renal lesions. These animals could be kept alive for weeks. The main histologic features were a disappearance of the beta cells from the islets, profound vacuolation of the pancreatic duct epithelium, and fatty changes of the liver. Doses of 25 mg. per kg. were without effect.

REFERENCES

ALLEN, F. M.: *J. Am. Med. Assoc.* 63: 939. 1914.
ALLEN, F. M.: *J. Metab. Res.* 1: 1. 1922.
BAUER, H.: *Zeitschr. f. mikrosk.-anat. Forsch.* 33: 143. 1933.
BRUNSCHWIG, A., AND J. G. ALLEN: *Cancer Research.* To be published.
DRAGSTEDT, L. R.: *Ann. Surg.* To be published.
DUNN, SHAW J., H. L. SHEEHAN AND N. G. B. MCLETCHIE: *Lancet* 1: 484. 1943.
EVANS, H. M., K. MEYER, M. E. SIMPSON AND F. L. REICHERT: *Proc. Soc. Exper. Biol. & Med.* 29: 857. 1931–32.
GOMORI, G.: *Proc. Soc. Exper. Biol. & Med.* 42: 23. 1939.
GOMORI, G.: *Am. J. Path.* 17: 395. 1941.
HAM, A. W., AND R. E. HAIST: *Am. J. Path.* 17: 787. 1941.
HOUSSAY, B. A., A. BIASOTTI AND C. T. RIETTI: *Compt. rend. soc. de Biol.* 111: 479. 1932.
JACOBS, H.: *Proc. Soc. Exper. Biol. & Med.* 37: 407. 1937.
MEHRING, J. VON, AND O. MINKOWSKI: *Arch. f. exp. Path. u. Pharm.* 26: 371. 1890.
RICHARDSON, K. C., AND F. G. YOUNG: *Lancet* 234: 1098. 1938.

STUDIES ON INACTIVATION OF ESTRADIOL BY THE LIVER[1]

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IT IS generally believed that estrogens are rapidly inactivated by the liver, this belief being based largely upon data obtained from four types of observation, *a*) Liver tissue (dog, rat, mouse, rabbit, guinea-pig, frog) and cell-free extracts of liver have been found to inactivate estrogens *in vitro* (Silberstein, *et al.*, 1933; Zondek, 1934; Engel and Navratel, 1937; Heller, *et al.*, 1939; Heller, 1940; Engel, 1941; Heller and Heller, 1943). *b*) It was reported (Israel, *et al.*, 1937) that estrone is not inactivated when perfused through a heart-lung preparation (dog) but is rapidly (within 15 minutes) inactivated when perfused through a heart-lung-liver preparation. *c*) Ovaries transplanted to the mesentery (Golden and Sevringhaus, 1942) or pellets of crystalline estrogen implanted in the spleen (Biskind and Mark, 1939; Biskind, 1940, 1941) exert little or no estrogenic effect as compared with their characteristic action when implanted subcutaneously. The same is true of estrogen injected into the spleen (Segaloff and Nelson, 1941). *d*) Enhancement of the effect of endogenous estrogen and of exogenous estrogens introduced into the systemic or portal circulations has been observed in animals and human subjects with hepatic damage (Talbot, 1939; Pincus and Martin, 1940; Glass, *et al.*, 1940; Biskind and Biskind, 1942; Biskind and Shelesnyak, 1942).

Estrone, injected into rabbits (Frank, *et al.*, 1932), rats (Zondek, 1934) and frogs (Engel and Navratel, 1937), disappears rapidly from the organism, little being excreted in the urine or feces. Zondek (1934) found that less than 5 per cent of the administered estrone could be extracted from the tissues in the free state after 3 hours and only 0.2 per cent after 20 hours. However, as much as 20 per cent could be recovered after hydrolysis. Critical analysis of the reported data reveals that the generally accepted views regarding the influence of the liver in this connection have been based upon surprisingly few observations and that there are significant gaps and contradictions in the chain of evidence that forms the basis for these views.

Data reported previously (Cantarow, *et al.*, 1942, 1943) indicate

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that the bile is an important medium of excretion of both exogenous and endogenous estrogen in human subjects and dogs. They also suggest the possibility of an entero-hepatic circulation of estrogen (Cantarow, *et al.*, 1943c). According to this hypothesis, the inactivity of estrogenic substances introduced into the portal field (peritoneum, mesentery, spleen, intestine) is due rather to its removal by the liver and excretion in the bile than to rapid destruction by the liver. It may subsequently undergo repeated absorption from the intestine and excretion in the bile, small amounts being perhaps lost in the feces (Dohrn and Faure, 1928; Siebke, 1940) and the bulk being gradually destroyed by the liver, very little or none escaping into the systemic circulation. This hypothesis is supported by the observation (Cantarow, *et al.*, 1943) of prolonged excretion (23 days) of comparatively large amounts of estrogen in the bile after implantation of a pellet of α-estradiol into the spleen, no estrogen appearing in the urine during this period. Estrogen was also excreted in the bile after instillation of α-estradiol into the duodenum. Because of the difficulty of reconciling these observations with the apparently rapid disappearance of estrogen from the organism (Zondek, 1934; Engel and Navratel, 1937), it was decided to study *a*) the distribution of estrogen in the blood and tissues following intravenous injection and *b*) the capacity of normal and damaged (CCl_4) liver tissue for inactivation of α-estradiol *in vitro*.

MATERIAL AND METHODS

Three types of experiment were performed. *a*) Two and five-tenths mg. (250,000 I.U.) of α-estrodiol[3] (in 0.5 cc. of absolute alcohol) were injected intravenously into 9 dogs. Eight were killed 3 to 48 hours later and the livers were assayed for free and conjugated estrogen. In one animal, under pentobarbital-sodium anesthesia, portions of the liver were removed for assay 10, 60 and 105 minutes after injection of the α-estradiol. *b*) Two and five-tenths mg. of α-estradiol were injected intravenously into 3 dogs. After 24 (2 animals) or 48 hours (1 animal), they were anesthetized with pentobarbital-sodium and blood was drawn for estrogen assay from the portal and hepatic veins, the femoral artery and, in one instance, from the inferior vena cava. The gall-bladder bile, liver, spleen and intestines were also assayed for estrogen. *c*) Liver tissue (0.25–25 gm.) from normal rats (10 experiments) and dogs (7 experiments) was reduced to a pulp, ground with sand and incubated with continuous shaking for 2 to 5 hours at 37° C. with 160 to 10,000 I.U. of α-estradiol in buffered Ringer solution at pH 6.4. Similar experiments were performed with the livers of rats that had received subcutaneous injections of 0.1 cc. of CCl_4 twice weekly for 8 to 11 weeks (7 experiments). At the end of the period of incubation the contents of the flasks were extracted for free and conjugated estrogen. For the free hormone, the contents of the flasks were extracted three times by mechanical shaking for 2 hours with equal volumes of ether. The ether extracts were pooled,

[3] We are indebted to Drs. Erwin Schwenk and Wm. H. Stoner of the Schering Corp., Bloomfield, N. J., for the estradiol used in this study.

evaporated and taken up in corn oil for biologic assay. For the conjugated hormone the residue was diluted with water and hydrolyzed at pH 1.0 (HCl) by boiling for 1.5 hours. This material was then extracted with ether and taken up in corn oil for assay.

All assays were made on young, adult mice of uniform size and age, 7 days after castration. The extracts were injected in 3 divided doses over a period of 3 days and vaginal smears were made at intervals during the next 48 hours. At least 6 animals were used at each dosage level. When the approximate potency of the extracts had been ascertained, the final assay was made using at least 4 closely graded dosages. If only small amounts of hormone were present, the assays were completed by sectioning the vaginas at 72 hours and reading according to the method of Fluhmann (1934).

RESULTS AND COMMENT

In vivo studies. The pertinent data are presented in the tables. Very little biologically active estrogen was demonstrable in the liver

TABLE 1. RECOVERY OF ESTROGEN FROM THE LIVER OF DOGS AFTER INTRAVENOUS INJECTION OF 2.5 MG. (250,000 I.U.) OF α-ESTRADIOL

Sex	Time	Liver Weight	Recovery			
			Free		Conjugated	
	hr.	*gm.*	I.U.	%	I.U.	%
F	24	310	186	.07		
M	3½	300	155	.06		
M	24	300	0	0	0	0
M	24	325	0	0	300	.12
M	24	200	0	0	0	0
M	48	447	0	0	0	0
M	24	450	120	.05	0	0
F	10 min.	78.5	492[1]	.2	492[1]	.2
	1	40.0	3960[1]	1.56	396[1]	.156
	105 min.	70.5	60[1]	.02	6[1]	.002
F	3	356	300	.12	180	.07

[1] Calculated on basis of total weight of remaining liver at time of removal of specimen for assay.

3 to 48 hours after intravenous injection of 250,000 I.U. of α-estradiol (table 1). This is in accord with previous observations in rats (Zondek, 1934) and frogs (Engel and Navratel, 1937). In the single experiment in which portions of the liver were removed at brief intervals after injection of estrogen, the quantity in the liver reached a maximum of 1.7 per cent of the amount injected at 60 minutes and fell to 0.03 per cent at 105 minutes. The significance of these data lies chiefly in their relation to the quantity of estrogen in the blood and bile at the same time intervals after its intravenous injection. These findings, reported elsewhere (Cantarow, *et al.*, 1942, 1943; Rakoff, *et al.*, 1943), indicate that large amounts of estrogen (20–48 per cent of the quantity administered) may be excreted in the bile of dogs during the second 24 hours after intravenous injection of 10,000 to 250,000 I.U. and 6 to 13

per cent more during the third 24 hours, even though none of the bile is returned to the animal. Moreover, estrogenic activity virtually disappears from the blood and urine after 24 hours in normal animals.

The peculiar situation is thus encountered in which estrogen continues to be excreted in the bile in comparatively large amounts for 24 to 48 hours after it has practically disappeared from the systemic blood and when little or none can be demonstrated in the liver. The data presented in table 2 were obtained in experiments designed to determine whether estrogen could be demonstrated in some portion of the portal field other than the liver. It was found that 24 and 48 hours after intravenous injection of 250,000 I.U. of α-estradiol the concentration of estrogen in the gall-bladder bile was extremely high,

TABLE 2. DISTRIBUTION OF ESTROGEN AFTER INTRAVENOUS INJECTION OF 2.5 MG. OF α-ESTRADIOL IN DOGS

Time after Injection	Gall-Bladder Bile		Liver		Portal Vein		Hepatic Vein		Femoral Artery		Inferior Cava		Spleen	Intestine
	Free	Total	Free	Total	Free	Total	Free	Total	Free	Total	Free	Total	Free	Free
hours	I.U.	I.U.	I.U.	I.U.	I.U.	I.U.	I.U.	I.U.	I.U.	I.U.	I.U.	I.U.	I.U.	
24	5000	0	0	0	300		0	0	100		50		0	
24	7500	0	26	26	0	0	0	0	0	0			0	
48	3000	0	0	0	0		0		0				0	0

while none could be demonstrated in the liver, spleen or intestine. In 2 of the 3 experiments no estrogenic activity was found in blood obtained from the portal vein, hepatic vein, inferior vena cava and femoral artery. In one instance, a comparatively large amount was demonstrated in the portal vein blood whereas none was found in the hepatic vein blood. The concentration in the portal vein blood was considerably higher than in the peripheral circulation. These findings suggest that estrogen is removed from the portal blood by the liver. There is no evidence that biologically active estrogen is stored anywhere in the portal field during the second 24 hours after its intravenous injection, and the biliary excretion of large amounts of estrogen during this period cannot be explained on this basis.

In Vitro studies. The results of incubation of estradiol with liver tissue are presented in table 3. There was a striking variation in the degree of inactivation by normal liver, ranging from 21 to 99 per cent for rat liver and 27 to 94 per cent for dog liver. The greater part of the recovered hormone was in the free state and neither the amount of liver, the amount of hormone nor the time of incubation had any consistent influence upon the extent of inactivation. This marked variation in inactivation is evident in data reported by Zondek (1934), but has not been emphasized in discussions of this subject. In 7 incubation experiments with rat liver and estrone, Zondek found 93 per cent loss of activity in 2 instances, 87 per cent loss in 3 and no loss in 2. These findings and the data reported here throw considerable doubt

upon conclusions based on quantitative differences observed in a limited series of experiments. There can be no doubt that both α-estradiol and estrone can be inactivated by liver tissue and by cell-free water extracts of liver (Zondek, 1934; Cantarow, *et al.*, unpublished observations). This property of liver tissue and extracts is shared by a num-

TABLE 3. *In vitro* INACTIVATION OF α-ESTRADIOL BY LIVER PULP

Amount Liver	Volume Solution	Time	Amount Hormone	Recovery	
				Free	Conj.
gm.	*cc.*	*hr.*	I.U.	%	%
		Normal rats			
0.25	5	5	160	6	3
1		2	200	15	
1		2	200	50	
1		4	200	50	
1		4	200	50	
8	30	4	5000	30	4
8	50	5	5000	66	13
8	200	4	6000	5	5
8	200	4	6000	5	5
24	50	4	10000	0.6	0
		Normal dogs			
0.25[1]	5	5	160	12.5	0
1[1]	30	5	1000	6	0
8[1]	30	5	5000	66	4
8[2]	30	2	3000	22	0.6
8[2]	30	2	3000	6.6	0.6
25[2]	50	2	5000	66	6.4
25[2]	50	5	5000	13	6.4
		Rats treated with CCl_4			
0.25	5	5	160	81	13
1	30	4	1000	10	0
8	30	5	5000	66	13
8	30	2	3000	22	0.6
8	50	2	5000	40	6.4
8	30	4	3000	22	2
8	50	4	5000	66	0

[1] Female.
[2] Male.

ber of substances, including hyacinth roots (von Euler and Zondek, 1934), potato juice, cauliflower, beet-roots, bran and certain bacteria (Zondek and Sklow, 1942), mushroom laccase and potato tyrosinase (Graubard and Pincus, 1942). It should be pointed out, however, that the data which constitute the basis for the current concept regarding the rôle of the liver in the intermediary metabolism of estrogens contain certain significant discrepancies.

Fee, Marrian and Parkes (1929), after perfusing heart-lung-kidney preparations with 500 cc. of blood containing 200 M.U. of estrone, could demonstrate no estrogenic activity in the perfused organs or perfusate. They suggested that the hormone might have been detroyed by oxidation in the lungs. Israel, Meranze and Johnston

(1937), on the other hand, in 2 heart-lung perfusion experiments, observed little if any inactivation of estrone. Addition of the liver to the perfusion system (3 experiments) resulted in rapid disappearance of estrogen from the perfusate (15 minutes), relatively insignificant amounts being recovered from the perfused organs at the end of 105 minutes. They concluded that estrone is inactivated by liver but not by lung. It should be noted that the kidney was included in the perfusion experiments of Fee, *et al.*, (1929) and that Zondek (1934) reported 20 per cent loss of estrogenic activity in 1 of 2 kidney-estrogen incubation experiments, whereas heart and lung were inactive in this respect. These data are particularly difficult to reconcile with the report by Heller (1940) that *in vitro* incubation of estrone with lung, heart and kidney substance results in an increase in estrogenic activity, perhaps by conversion of estrone to estradiol. Incubation with spleen tissue (Heller, 1940) also caused an increase in estrogenic activity, in contrast to the report by Zondek (1934) of 20, 25, and 0 percentage loss of activity in 3 similar experiments. Further confusion is added by the observation of Engel and Navratel (1937) that practically the same amount (93%) of estrogen disappeared from the bodies of intact and hepatectomized frogs during a period of 48 hours after injection into the lymph sac.

Attempts have been made to determine the identity of the enzyme responsible for the *in vitro* inactivation of estrogen by liver tissue. According to Zondek and Sklow (1942), the enzyme is destroyed in 1 hour by 2 per cent H_2O_2, has an optimum pH range of 6 to 7, is rendered ineffective by M/500 NaCN solution and has properties similar to but not identical with those of tyrosinase; *e.g.*, cauliflower, which has no tyrosinase, inactivates estrogen while mealworm larvae, rich in tyrosinase, do not. Graubard and Pincus (1942) were unable to demonstrate any appreciable oxygen uptake when water-soluble estrogen esters were added to rabbit liver, with and without added cytochrome. They suggest that esterification rather than oxidation may be responsible for inactivation of estrogen by the liver, a possibility which is not supported by the application of the usual hydrolysis procedures for demonstrating esterified estrogens. This possibility cannot, however, be definitely excluded on this basis.

Several observers have demonstrated increased effectiveness of endogenous and exogenous estrogens in the presence of hepatic damage (Talbot, 1939; Pincus and Martin, 1949; Glass, *et al.*, 1940; Biskind and Biskind, 1942; Biskind and Shelesnyak, 1942). This has been attributed to decreased inactivation of estrogen by the functionally damaged liver cells, but we are unaware of any reported observations that damaged liver tissue is less potent than normal liver in inactivating estrogen *in vitro*. The data presented in table 3 reveal no significant difference between normal and damaged liver in this regard.

Histological examination showed characteristic acute and chronic changes in the livers of the rats treated with CCl_4.

These data do not support the hypothesis that the increased estrogenic activity observed in experimental animals and human subjects with hepatocellular damage is dependent upon diminished capacity of the liver cells for inactivating estrogen. It is possible, in advanced cirrhosis of the liver, with distortion of the lobular architecture that a portion of the blood coming to the liver may be shunted directly into the hepatic vein radicles without coming into intimate contact with functioning liver cells, with consequent inadequate opportunity for inactivation of estrogen. However, this anatomical situation is not encountered in acute hepatic damage nor in mild grades of fibrosis, and cannot explain many of the reported observations in experimental animals and human subjects. If prompt removal by the liver and excretion in the bile are responsible for the rapid disappearance of estrogenic activity from the peripheral circulation, impairment of these functions incident to hepatocellular functional damage can explain satisfactorily the enhancement of estrogenic activity observed under such circumstances. Prolongation of the curve of blood estrogen following administration of estrogen to patients with acute and chronic hepatic damage (Rakoff, *et al.*, 1943) would thus be due to diminished removal and excretion by the liver rather than to diminished inactivation or destruction by that organ The excretion of comparatively large amounts of exogenous estrogen in the bile during periods of time when little or none can be demonstrated in the urine, blood or tissues, including the liver, can be explained satisfactorily by assuming that it is converted in the liver to a substance possessing little or no estrogenic activity, is in part probably stored temporarily in this form in the liver and is 'reactivated' during the process of its excretion by the liver cells. No other premise seems adequate to explain all of the data now available

In view of the presence of only small amounts of estrogen in the feces and the direct demonstration of absorption of estrogen from the intestine and its subsequent excretion in the bile, it seems probable that estrogens undergo an enterohepatic circulation similar to that of bile acids. This phenomenon removes them from the systemic circulation, as it does bile acids, practically as effectively as would rapid destruction in the liver. The effect of hepatic damage in prolonging the increase in bile acid in the blood and urine after administration of bile acids bears a striking resemblance to its effect upon blood and urine estrogen after estrogen administration.

It is probable that the estrogen is gradually destroyed or converted to a permanently inactive substance, but the hypothesis that this occurs rapidly under physiological conditions is contradicted by the results of our studies.

SUMMARY

Little or no active estrogen can be recovered from the liver of the dog 3 to 48 hours after intravenous injection of 250,000 I.U. of α-estradiol. Twenty-four and 48 hours after injection, large amounts are present in the gall-bladder bile, but none in the liver, spleen, intestinal wall and hepatic vein blood. In 1 of 3 experiments there was a relatively high concentration of estrogen in the portal vein blood.

The livers of rats poisoned with CCl_4 exhibit essentially the same capacity as normal rat liver for inactivating α-estradiol *in vitro*. These observations do not support the hypothesis that the increased activity of endogenous and exogenous estrogen in the presence of hepatic damage is due to decreased inactivation of estrogen by the damaged liver cells.

The following hypothesis is in accord with all available data. Estrogen is removed from the blood by the liver cells, is converted to a substance possessing little or no estrogenic activity, is in part stored temporarily in the liver in this form and is 'reactivated' during the process of its excretion by the hepatic cells. The estrogen probably undergoes an enterohepatic circulation similar to that of bile acids, being perhaps gradually destroyed or converted to a permanently inactive substance.

REFERENCE

BISKIND, G. R.: *Proc. Soc. Exper. Biol. & Med.* 43: 259. 1940; 47: 266. 1941.
BISKIND, M. S., AND G. R. BISKIND: *Endocrinology* 31: 109. 1942.
BISKIND, G. R., AND J. MARK: *Bull. Johns Hopkins Hosp.* 65: 212. 1939.
BISKIND, M. S., AND M. C. SHELESNYAK: *Endocrinology* 30: 819. 1942.
CANTAROW, A., A. E. RAKOFF, K. E. PASCHKIS, L. P. HANSEN AND A. A. WALKLING: *Proc. Soc. Exper. Biol. & Med.* 49: 707. 1942a.
CANTAROW, A., A. E. RAKOFF, K. E. PASCHKIS, L. P. HANSEN AND A. A. WALKLING: *Endocrinology* 31: 515. 1942b.
CANTAROW, A., A. E. RAKOFF, K. E. PASCHKIS, L. P. HANSEN AND A. A. WALKLING: *Proc. Soc. Exper. Biol. & Med.* 52: 256. 1943c.
DOHRN, M., AND W. FAURE: *Klin. Wchnschr.* 7: 943. 1928.
ENGEL, P.: *Endocrinology* 29: 290. 1941.
ENGEL, P., AND E. NAVRATEL: *Biochem. Ztschr.* 292: 434. 1937.
v. EULER, H., AND B. ZONDEK: *Biochem. Ztschr.* 271: 64. 1934.
FLUHMANN, C. F.: *Endocrinology* 18: 705. 1934.
FRANK, R. T., M. A. GOLDBERGER AND F. SPIELMAN: *Proc. Soc. Exper. Biol. & Med.* 29: 1229. 1932.
GLASS, S. J., H. A. EDMONDSON AND S. N. SOLL: *Endocrinology* 27: 749. 1940.
GOLDEN, J. B., AND E. L. SEVRINGHAUS: *Proc. Soc. Exper. Biol. & Med.* 39: 361. 1938.
GRAUBARD, M., AND G. PINCUS: *Endocrinology* 30: 265. 1942.
HELLER, C. G.: *Endocrinology* 26: 619. 1940.
HELLER, C. G., AND E. J. HELLER: *Endocrinology* 32: 64. 1943.
HELLER, C. G., E. J. HELLER AND E. L. SEVRINGHAUS: *Am. J. Physiol.* 126: P530. 1939.
ISRAEL, S. L., D. R. MERANZE AND C. G. JOHNSTON: *Am. J. Med. Sci.* 194: 835. 1937.
PINCUS, G., AND D. W. MARTIN: *Endocrinology* 27: 838. 1940.
RAKOFF, A. E., PASCHKIS, K. E., A. CANTAROW, L. P. HANSEN AND A. A. WALKLING: submitted for publication.
SEGALOFF, A., AND W. O. NELSON: *Proc. Soc. Exper. Biol. & Med.* 48: 33. 1941.
SIEBKE, H.: *Zentralbl. f. Gynäk.* 54: 1734. 1940.
SILBERSTEIN, F., P. ENGEL AND K. MOLNAR: *Klin. Wchnschr.* 12: 1693. 1933.
TALBOT, N. B.: *Endocrinology* 25: 601. 1939.
ZONDEK, B.: *Skand. Arch. f. Physiol.* 70: 133. 1934.
ZONDEK, B., AND J. SKLOW: *Proc. Soc. Exper. Biol. & Med.* 49: 629. 1942.

EFFECTS OF ESTRONE, ASCORBIC ACID AND TESTOSTERONE PROPIONATE ON NITROGEN STORAGE AND INSULIN REQUIREMENT IN DOGS

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THE early experiments with estrogens in depancreatized animals dealt primarily with effects of these substances upon glycosuria and time of survival when treatment with insulin was omitted or discontinued (Barnes, *et al.*, 1933; Nelson and Overholser, 1936; Collip, *et al.*, 1937). We became interested in studying effects of estrone in depancreatized dogs kept as nearly normal as possible by means of diet and insulin treatment. Such animals show small spontaneous fluctuations in their insulin requirement, and are in rather delicate equilibrium. This is particularly evident if one studies numerous criteria simultaneously, *e.g.*, body weight, water intake, urine volume, the output of glucose, ketones and nitrogen, and the concentration of sugar and fat in the blood. Whether estrone, given in addition to the maintenance dose of insulin, counteracts any of the effects of anterior pituitary preparations in these animals also seemed of interest. That estrone alone would not completely prevent the increase in glycosuria was shown in previous experiments by Nelson and Overholser (1936). Besides giving estrone with insulin, we therefore set up controlled experiments to detect any reduction of the glycosuria, or prevention of the nitrogen loss, which is more easily controlled (Gaebler and Galbraith, 1941).

Identical experiments were made with ascorbic acid. We suspected that the unfavorable effects of growth preparations in pancreatic diabetes might be due in part to injection of foreign protein, and the numerous reports that ascorbic acid prevents certain allergic and anaphylactic reactions accounted for our interest in this compound.

Since testosterone propionate is a pure substance which produces nitrogen storage (Kochakian and Murlin, 1935; 1936; Thorn and Engel, 1938; Kenyon, *et al.*, 1940; Knowlton, *et al.*, 1942), a comparison of its effects, in normal and depancreatized animals, with those of anterior pituitary growth preparations previously studied in this laboratory was desired. In normal animals single large doses of growth preparations cause sudden increases in water intake and body weight, marked storage of nitrogen, and a large calorigenic effect, but no apparent derangement of metabolism. In depancreatized animals receiving a constant dose of insulin they produce nitrogen loss, and marked glycosuria, accompanied by ketosis and lipemia. The intensity of these effects seems to parallel the potency of the preparations

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for promoting growth (Shipley and Long, 1938) or nitrogen storage (Gaebler and Robinson, 1942). That testosterone propionate moderately increases glycosuria in partially depancreatized ferrets has been reported by Dolin, Joseph and Gaunt (1941), who also cite papers in which reduction of glycosuria with androgens was reported. In the present studies completely depancreatized dogs were used, and observations on urine nitrogen, blood fat, and other metabolites were made simultaneously.

EXPERIMENTAL METHODS

Adult bitches which had lived in metabolism cages for at least two months were used. Experiments on the two depancreatized ones covered a 6-month period beginning 3 to 4 weeks after the operation. Upon withdrawal of insulin, the D:N ratio, extent of ketosis and other criteria indicated complete pancreatectomy in both subjects. This was also confirmed in one of the animals by autopsy; the other one is still being used.

A diet consisting chiefly of raw pancreas and casein was used almost exclusively. To assure its constancy in a given set of experiments, a large amount was mixed in a mechanical dough mixer and weighed out in packages which were wrapped in paraffined paper and frozen until needed. Each package contained 125 gm. of trimmed raw pancreas, 50 gm. of commercial casein, 12.5 gm. of dibasic calcium phosphate, and 2.5 or 5.0 gm. of salt mixture (Karr, 1920). Depancreatized animals received a package of this diet at 9 A.M., and another at 5 P.M. One normal animal (dog 35, table 3) received three-fourths as much. All of these animals also received 15 drops of haliver oil and 2.5 gm. of compressed yeast in tablet form daily. In one experiment (dog 34, table 3) we used a diet of casein, cracker meal, corn oil, yeast and salt mixture. Haliver oil and a measured amount of water were added before feeding.

Pancreatic diabetes was controlled by injecting protamine insulin once or twice daily, and supplementing it with plain insulin after meals. Weighed amounts of an anterior pituitary preparation[1] were dissolved in 0.02 N sodium hydroxide and injected subcutaneously after partial neutralization.

All animals were catheterized at the same hour each morning, and were then weighed. Water intake and urine volume were measured daily. The analytical methods were the same as in previous studies reported from this laboratory. Blood sugar values represent true sugar.

RESULTS

Estrone. Two depancreatized animals were used for the experiments shown in table 1. The growth preparation (G-1) was injected subcutaneously, on the first day of the experimental periods in which it was given, the dose being 100 mg. Estrone (Es), whether given alone or in the same period as the growth preparation, was injected daily throughout the period, the daily dose being 1 cc. or 10,000 I.U. in oil. During the first experiment with dog 32, a complete nitrogen balance study was made, but only the urine nitrogen is recorded. For the pe-

[1] The anterior pituitary preparation (Antuitrin, Growth) was kindly supplied in powder form by Parke, Davis and Co., Detroit, Mich.

riod when the growth preparation was given, the positive balance was 1.43 gm. per day; during the preceding and following control periods the average positive balance was 1.60 gm. per day. With the insulin dose constant, the growth preparation thus had its usual effect in a depancreatized animal—it failed to improve nitrogen storage, and caused marked glycosuria, hyperglycemia and some increase in blood

TABLE 1. EFFECTS OF A GROWTH PREPARATION AND ESTRONE, SEPARATELY AND TOGETHER, IN TWO DEPANCREATIZED ANIMALS RECEIVING CONSTANT DIETS

Animal and Date	Procedure[1]	Insulin per Day	Wt.	Average of Daily Urine Analyses			Composition of Blood per 100 cc.		
				Nitrogen	Sugar	Ketones	Average NPN	Average sugar	Maximum fat
1941–2		*units*	*kg.*	*gm.*	*gm.*		*mg.*	*mg.*	*mg.*
Dog 32									
11/21–23	Control	24+12[2]	12.8	16.85	0.0	−	42	180	829
11/24–26	G-1	24+12	12.8	17.03	34.2	+	42	301	1073
11/27–28	Control	24+12	13.0	17.33	2.5	−	44	277	850
11/29–12/13	Interval								
12/14–16	Control	10+10	13.0	20.67	1.7	−	42	209	1054
12/17–20	Es	10+10	13.0	19.24	2.2	−	40	205	
12/21–26	Es	10+10	13.3	19.00	0.0	−		244	1071
12/27–29	Es & G-1	10+10	13.4	19.42	79.4	+	34	281	2087
12/30–1/2	Control	10+10	13.0	19.48	14.9	−	38	171	776
Dog 33									
12/19–22	Control	8+8	12.2	19.73	6.7	−	42	236	1121
12/23–26	Es	8+8	12.2	19.39	2.6	−	38	235	1095
12/27–28	Es & G-1	8+8	12.3	23.42	89.9	+	37	290	1901
12/30–1/1	Control	8+8	11.9	19.18	27.7	−	45	256	825
1/2–2/22	Interval								
2/23–2/25	Control	12+8	11.3	17.81	0.0	−	27	56	695
2/26–28	G-1	12+8	11.1	19.16	42.7	+	30	167	710
3/1–3	Control	12+8	11.3	18.34	0.0	−		38	

[1] Es and G-1 designate estrone and growth preparation, respectively; doses and duration of treatment are given in the text. Both animals received the same amount of the pancreas-casein diet, containing 20.0 gm. of nitrogen per day. Zero values for urine sugar indicate a negative qualitative test.

[2] First figure, protamine insulin; second figure, plain insulin.

fat. The result was the same in the other experiment with growth preparation alone, recorded at the foot of table 1.

A barely significant decrease in urine nitrogen followed administration of estrone alone. In the experiment on dog 32 the large decrease from 20.67 gm. to 19.24 gm. per day may include a spontaneous change, since the output during the final control period was 19.48 gm. per day. When estrone and the growth preparation were given together, one animal showed a slight increase in nitrogen output and the other a decided increase. In both animals the glycosuria and lipemia were much more marked than in the experiments with the growth preparation alone.

It is quite evident that none of the effects of the pituitary preparation were counteracted by estrone. Whether estrone intensified the diabetic syndrome still further is open to question, for in each animal

TABLE 2. EFFECTS OF A GROWTH PREPARATION AND ASCORBIC ACID, SEPARATELY AND TOGETHER, IN TWO DEPANCREATIZED ANIMALS RECEIVING A CONSTANT DIET

Animal and Date	Procedure[1]	Insulin per Day	Wt.	Average of Daily Urine Analyses			Composition of Blood per 100 cc.		
				Nitrogen	Sugar	Ketones	Average NPN	Average sugar	Maximum fat
1942		*units*	*kg.*	*gm.*	*gm.*		*mg.*	*mg.*	*mg.*
Dog 32									
2/13–15	Control	12+8[2]	13.5	19.55	0.0	–	41	98	862
2/16–18	G-1 & C	12+8	13.4	19.74	26.7	–	43	220	840
2/19–20	Control	12+8	13.6	18.77	3.1	–	38	42	974
2/21–22	Interval								
2/23–26	Control	11+8	13.9	18.90	0.0	–	33	62	784
2/27–3/1	G-2	11+8	13.6	20.70	23.3	–	34	143	1125
3/2–4	Control	11+8	13.7	19.17	0.0	–	39	30	
3/5–10	Interval								
3/11–13	Control	10+8	13.9	19.21	0.0	–	35	79	1007
3/14–16	G-2 & C	10+8	13.5	20.43	49.5	–	33	221	1408
3/17–19	Control	10+8	13.6	18.92	8.9	–	35	60[3]	1035
3/20–23	Interval								
3/24–26	Control	9+7	13.7	18.18	3.0	–	38	70	877
3/27–29	C	9+7	13.7	17.71	5.5	–	36	146	945
3/30–4/1	Control	9+7	13.7	18.86	6.3	–	37	130	846
Dog 33									
2/23–25	Control	12+8	11.3	17.81	0.0	–	27	65	695
2/26–28	G-1	12+8	11.1	19.16	42.7	+	30	167	728
3/1–3	Control	12+8	11.3	18.34	0.0	–		38	
3/4–23	Interval								
3/24–26	Control	10+2	11.3	18.31	0.0	–	37	49	
3/27–29	G-1 & C	10+2	11.1	19.13	51.3	+	27	208	
3/30–4/1	Control	10+2	11.2	18.82	19.9	–	36	73	
4/2–4/29	Interval								
4/30–5/3	Control	11+4	11.5	18.97	0.0	–	34	87	731
5/4–6	C	11+4	11.6	18.32	0.0	–	30	41	704
5/7–10	Control	11+4	11.7	18.55	0.0	–	38	36	720

[1] G-1 and G-2 designate two lots of growth preparation; C designates ascorbic acid. The doses and duration of treatment are given in the text. Both animals received the same amount of the pancreas-casein diet, containing 20.84 gm. of nitrogen per day. Zero values for urine sugar indicate a negative qualitative test.

[2] First figure, protamine insulin; second figure, plain insulin.

[3] Value on last day of period only.

the dose of insulin was lower during the experiment with estrone plus growth preparation than during the one with the growth preparation alone. In dog 32 this was due to a diminished insulin requirement. The first experiment was begun during the fourth week after pancreatectomy, when 36 U of insulin per day was still required to abolish glycosuria. This amount permitted hyperglycemia to occur at the end of the 24-hour period, when the blood-sugar determinations were made. During the control period beginning December 14, the same degree of control was obtained with only 20 U of insulin, the diet being unchanged. In the case of dog 33, the insulin doses in the two experiments differed by only 4 U per day, but comparison of the control periods shows that this caused a change from the borderline of glycosuria to that of hypoglycemia. The dose was increased because we feared

that a second diabetic episode as severe as the first one in this animal would cause vomiting.

Ascorbic acid. The same two animals were used in 7 experiments recorded in table 2. The growth preparations G-1 and G-2 were injected subcutaneously, in single 100-mg. doses, on the first day of periods in which they were given. Ascorbic acid (C), whether given alone or with the growth preparation, was given orally in 100-mg. doses, 3 doses on the first, 6 on the second, and 3 on the third day of the period. This distribution was made because metabolic effects of the growth preparation are always greatest during the second 24-hour period after injections.

In all experiments with growth preparations G-1 and G-2 the urine nitrogen rose slightly and the weight fell slightly. This is the characteristic effect in depancreatized animals receiving a constant dose of insulin, and it was not materially altered by giving ascorbic acid with the growth preparations. There is some evidence that these large doses of ascorbic acid promoted nitrogen storage, for the urine nitrogen fell slightly in the two experiments with ascorbic acid alone, and did not rise as much when ascorbic acid was given with the growth preparation G-2 as when the latter was given alone.

Ascorbic acid alone appears to have had no effect on the insulin requirement. The two experiments with the ascorbic acid alone were made under particularly favorable circumstances. As the urine and blood-sugar values indicate, dog 32 was slowly developing glycosuria and hyperglycemia, while dog 33 was gradually becoming hypoglycemic, and neither trend was influenced by ascorbic acid. In the first experiment on dog 32, with growth preparation G-1 and ascorbic acid, the glycosuria was smaller than in the experiment on dog 33, with G-1 alone. But in each animal one of the two growth preparations was given alone and with ascorbic acid, and in each case the glycosuria was larger when ascorbic acid was given.

Doses of the growth preparation which would interrupt observations were avoided, and effects upon urine ketones and blood fat were therefore small. In the pair of experiments with growth preparation G-2, blood fat rose slightly whether or not ascorbic acid was given, and in the corresponding pair of experiments with growth preparation G-1 ketones appeared in the urine in both instances.

Testosterone propionate. Normal bitches were used for the two experiments recorded in table 3. Testosterone propionate[2] (TP) was given intramuscularly in sesame oil solution, 25 mg. daily, for 4 days in one animal and 6 days in the other. The urine nitrogen fell in both experiments, and in the one in which a complete balance study was made the positive balance increased. The fall in urine nitrogen was not due to an increase in blood non-protein nitrogen. In these respects our

[2] For part of the testosterone propionate we are indebted to the Schering Corporation, Bloomfield, N. J.

TABLE 3. EFFECTS OF TESTOSTERONE PROPIONATE IN TWO NORMAL DOGS RECEIVING CONSTANT DIETS

Animal and Date	Procedure[1]	Average Values per Day					Composition of Blood per 100 cc.		
		Water intake	Urine volume	Wt.	Urine N	N Balance	NPN	Sugar	Fat
1942		*cc.*	*cc.*	*kg.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*
Dog 34									
5/15–20	Control	727	442	16.4	9.98		37	58	651
5/21–24	TP	740	392	16.3	8.90		37	63	650
5/25–31	Control	953	584	16.3	9.85		38		655
Dog 35									
9/25–27	Control	742	510	13.9	14.31	+0.04	39	65	732
9/28–30	Control	887	540	13.9	13.69	+0.66	38	66	737
10/1–3	TP	785	543	13.9	14.17	+0.05	40	68	720
10/4–6	TP	797	572	13.9	13.24	+1.17	34	67	720
10/7–9	Control	823	687	14.0	13.76	+0.53	36	67	705
10/10–12	Control	793	627	13.8	14.52	−0.28	36	68	639

[1] TP signifies testosterone propionate; dog 34 received 25 mg. daily for 4 days, and dog 35 received 25 mg. daily for 6 days. The water intake recorded includes water present in the food or available by oxidation. In the case of dog 35, diet, urine and stool were all analyzed. The nitrogen intake of dog 34 was approximately 9.7 gm. per day.

results resembled those of Kochakian and Murlin (1935; 1936) in castrated animals. The nitrogen storage was much smaller than that which was observed by Thorn and Engel (1938) in an experiment in which testosterone propionate was given to a normal male dog, and was scarcely a fourth as great as that which we regularly produce in bitches with growth preparations. The prominent effects of the latter on weight, water intake and urine volume were also completely absent in our experiments with testosterone propionate.

Two similar experiments in depancreatized bitches receiving constant diets and constant doses of insulin are shown in table 4. Each animal received 25 mg. of testosterone propionate for 6 days. Again there was no effect on water intake, urine volume or weight. The urine

TABLE 4. EFFECTS OF TESTOSTERONE PROPIONATE IN TWO DEPANCREATIZED ANIMALS RECEIVING CONSTANT DIETS

Animal and Date	Procedure[1]	Insulin per Day	Average Values per Day						Composition of Blood per 100 cc.		
			Water intake	Urine volume	Weight	Urine N	N Balance	Urine sugar	NPN	Sugar	Fat
1942		*units*	*cc.*	*cc.*	*kg.*	*gm.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*
Dog 32											
4/29–5/3	Control	13+7	1552	1138	14.5	19.67		0	39	48	889
5/4–9	TP	13+7	1653	1213	14.5	18.51		0	38	84	866
5/10–17	Control	13+7	1520	1108	14.8	19.60		2.5[2]	40	110	855
Dog 33											
8/5–7	Control	8+2	1339	893	11.7	16.72	+1.40	0	31	78	712
8/8–10	Control	8+2	1291	1003	11.8	17.89	+0.29	3.5	27	184	743
8/11–13	Control	8+4	1464	1043	11.9	17.42	+0.79	2.4	35	202	683
8/14–16	TP	8+4	1774	1267	12.0	17.64	+0.12	5.1	34	253	732
8/17–19	TP	8+4	1062	987	12.1	16.71	+0.92	2.0	32	197	711
8/20–22	Control	8+4	2029	1390	12.0	18.87	−1.05	21.9	29	128	678

[1] TP signifies testosterone propionate, of which each animal received 25 mg. daily for 6 days. In the case of dog 33 the diet, urine and stools were analyzed. The nitrogen intake of dog 32 was approximately 20.8 gm. per day. Water intake recorded includes water present in the food or available by oxidation. Insulin dosage is recorded in units of protamine and plain insulin, respectively.

[2] 20.2 gm. of glucose were excreted on the first day of this period.

nitrogen fell in both experiments, but in the second one neither the urine nitrogen nor the balance indicated storage greater than occurred spontaneously in the first control period. No glycosuria was produced. In connection with the blood true sugar values it must be emphasized again that these represent values reached for a short time at the end of the 24-hour period. Their trend was quite independent of the injections of testosterone propionate. Blood fat was unaffected. Thus testosterone propionate, which produces some nitrogen storage, had none of the unfavorable effects upon pancreatic diabetes that growth preparations have. A striking effect which it did have is obscured in the condensed tables, which give only the average values per day for periods of some length. In both experiments a large output of glucose in the urine occurred on the first day after injections of testosterone propionate were discontinued—20.2 gm. of glucose in the first experiment and 54.7 gm. in the second.

DISCUSSION

The original experiments of Barnes, Regan and Nelson (1933) were based on the theory that the effect of hypophysectomy upon diabetes, which they had confirmed, might be simulated by inhibiting the diabetogenic action of the pituitary with estrogens. Our experiments have no direct bearing on this theory, for our animals were treated with insulin and were not castrated. Inhibition of the pituitary by hormones might already have been at its maximum. The fact that Collip, Selye and Neufeld (1937) obtained no evidence for the theory might also be explained by their statement that in the species of monkey which they used the diabetes resulting from pancreatectomy already resembled that of hypophysectomized-depancreatized animals in some respects. The present experiments do however show that estrone produces no insulin sensitivity, which is such an outstanding characteristic of hypophysectomized animals, and that it does not counteract any of the effects of anterior pituitary preparations in depancreatized bitches.

Since our experiments with ascorbic acid were negative, they are of no interest in connection with the question of whether any of the effects of anterior pituitary preparations upon diabetes are due to injection of foreign protein. They are of interest in connection with the systematic study of effects of constituents of the adrenal cortex upon pancreatic diabetes, and in connection with the use of mixtures of water-soluble vitamins including ascorbic acid in the treatment of diabetes. Since the dog does not require exogenous ascorbic acid, a deficiency did not exist in our experiments. The excess of ascorbic acid did not necessitate reduction of the insulin dose, and prevented none of the effects of anterior pituitary preparations. Whether it permitted any reduction of the insulin dose was not investigated.

Previous investigators (Kochakian and Murlin, 1935; 1936; Kenyon, *et al.*, 1940) have pointed out that the effects of testosterone de-

rivatives on nitrogen storage may be absent if gonadal function is adequate. Our experiments add another instance in which positive, though small, nitrogen storage has been produced in normal animals. Since the effect was meager, a large series of experiments would be required to determine whether removing the pancreas and keeping the insulin supply constant limits the effect of the androgen on nitrogen storage still further.

The large glycosuria on the first day after withdrawal of testosterone propionate was accompanied by a rise in nitrogen output of less than 1 gm. per 12 gm. of glucose, and can therefore not be explained on the basis that withdrawal of the androgen removed a factor inhibiting gluconeogenesis from protein.

SUMMARY

Depancreatized bitches kept as nearly normal as possible by means of an adequate diet containing a large amount of raw pancreas, and by treatment with insulin, were used as subjects. Injection of 10,000 I.U. of estrone daily for 4 to 10 days, or feeding of 1.2 gm. of ascorbic acid in 3 days, was accompanied by slight diminution of the nitrogen output, but no indication whatever of increased sensitivity to insulin. Neither of the substances prevented the nitrogen loss, glycosuria, lipemia or ketonuria caused by administration of anterior pituitary preparations. In fact, additional experiments would be required to determine whether or not the diabetic syndrome was intensified still further.

In normal bitches the nitrogen storage produced by testosterone propionate, in doses of 25 mg. daily for 4 to 6 days, developed more slowly and was far less extensive than that which follows injection of anterior pituitary growth preparations. Testosterone propionate, however, induced slight storage of nitrogen in depancreatized bitches receiving a constant dose of insulin, and caused no glycosuria, ketonuria or lipemia. Cessation of injections was accompanied by marked glycosuria on the first day, quite out of proportion to the rise in nitrogen output.

REFERENCES

Barnes, B. O., J. F. Regan and W. O. Nelson: *J. Am. Med. Assoc.* 101: 926. 1933.
Collip, J. B., H. Selye and A. Neufeld: *Am. J. Physiol.* 119: 289. 1937.
Dolin, G., S. Joseph and R. Gaunt: *Endocrinology* 28: 840. 1941.
Gaebler, O. H., and H. W. Galbraith: *Endocrinology* 28: 171. 1941.
Gaebler, O. H., and A. R. Robinson: *Endocrinology* 30: 627. 1942.
Karr, W. G.: *J. Biol. Chem.* 44: 255. 1920.
Kenyon, A. T., K. Knowlton, I. Sandiford, F. C. Koch and G. Lotwin: *Endocrinology* 26: 26. 1940.
Knowlton, K., A. T. Kenyon, I. Sandiford, G. Lotwin and R. Fricker: *J. Clin. Endocrinol.* 2: 671. 1942.
Kochakian, C. D., and J. R. Murlin: *J. Nutrition* 10: 437. 1935.
Kochakian, C. D., and J. R. Murlin: *Am. J. Physiol.* 117: 642. 1936.
Nelson, W. O., and M. D. Overholser: *Endocrinology* 20: 473. 1936.
Shipley, R. A., and C. N. H. Long: *Biochem. J.* 32: 2242. 1938.
Thorn, G. W., and L. L. Engel: *J. Exper. Med.* 68: 299. 1938.

EFFECTS OF ADRENAL CORTICAL COMPOUNDS ON LACTATION[1]

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EARLIER studies have shown that activity of the adrenal cortex as well as the hypophysis is necessary for the initiation and maintenance of lactation in the guinea pig (Nelson and Gaunt, 1936, 1937a, 1937b; Gomez and Turner, 1936, 1937).

In the rat adrenalectomy does not abolish lactation immediately, but it is necessary in most cases that replacement therapy be given to obtain a secretion of milk adequate to maintain the lives of suckling young; and such therapy is necessary in all cases to support normal growth (Literature cited by Gaunt, Eversole and Kendall, 1942).

The availability of crystalline adrenal steroids has led to an examination of their capacity to support lactation in adrenalectomized animals. It has been shown (Gaunt, Eversole and Kendall, 1942) that in the adrenalectomized rat 1 mg. daily of 17-hydroxy-11-dehydrocorticosterone (Compound E of Kendall) would maintain normal lactation. Dehydrocorticosterone (Compound A) was almost if not equally as effective. Desoxycorticosterone acetate showed only weak activity and in no case supported a fully normal lactation.

In the studies reported here the hypophysectomized guinea pig has been used as the principal test object for a further study of the adrenal steroids in relation to the induction of lactation.

METHODS

Young guinea pigs, of either sex and weighing from 300 to 450 gm., were prepared for lactation by daily injection with estrogen (100 I.U.) for 25 or 30 days. It has been shown in earlier experiments that under this treatment the mammary glands develop and lactation occurs spontaneously when injections are discontinued. If hypophysectomy is performed at or soon after the time of estrogen withdrawal, lactation does not occur in the absence of appropriate replacement therapy. In these experiments treatment with estrogen was suspended 2 or 3 days before hypophysectomy. Post-operative care in-

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cluded the administration of sugar solutions and the feeding of tomato juice and greens *ad libitum*. Treatment with pituitary lactogen and the other substances being tested was begun within 24 hours after hypophysectomy. At the termination of each experiment the animal was autopsied and a careful search was made for remnants of the hypophysis. Suspected material was saved together with the adrenals, gonads, thyroids and mammary glands for histological study.

The lactogenic hormone preparations were prepared in the Parke, Davis & Co. laboratories and with one exception were supplied in the form of a powder. They were prepared for injection in a buffer solution, as were the adrenotropic fractions.[2]

The adrenal cortical substances,[3] with the exception of aqueous cortical extract, were dissolved in peanut or sesame oil and injected subcutaneously. Compound E was first dissolved in minimal amounts of acetone and sesame oil added to desired volume.

All treatments were given in divided doses twice daily. The secretion of milk was estimated semi-quantitatively and recorded as follows: 1+, trace; 2+, scanty; 3+, good; 4+, copious.

RESULTS

The results are summarized in table 1, the various experiments being divided into groups based largely on the particular lactogenic preparation used. This division is necessary since the preparations of lactogen varied in the amount of adrenotropic hormone[4] they contained, a factor of importance in determining the induction of lactation.

Experiments with lactogen 906 910-A (*Series 1*). This preparation was supplied in solution and contained an appreciable amount of adrenotropic hormone. There was enough of the latter present that some lactation could be induced in some of the hypophysectomized animals with only 2 or 3 cc. of the material. Lactation could not be improved, however, if it occurred at all, by adding desoxycorticosterone to the therapy. Cortical extract, on the other hand, improved the response to the lactogen markedly, and the same was true to a lesser extent in the two cases in which the lactogen and 17-hydroxy-11-dehydrocorticosterone were given together.

Experiments with lactogen 1034C (*Series 2*). This was an excellent

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[2] We are very grateful to Dr. Oliver Kamm, Parke, Davis & Company for supplying us with the lactogenic and adrenotropic hormones and for the adrenal cortical extract (Eschatin) used in these studies.

[3] We should like to acknowledge the kindness of Dr. Erwin Schwenk, Schering Corporation, Bloomfield, N. J., and of Dr. Ernest Oppenheimer, Ciba Pharmaceutical Products, Inc., Summit, N. J., for furnishing desoxycorticosterone acetate and of Dr. E. C. Kendall, Mayo Foundation, for supplying 17-hydroxy-11-dehydrocorticosterone.

[4] The assay values for adrenotropic activity, on the basis of the total amounts required to double the weight of adrenals in hypophysectomized immature rats, were 120 mg. for 1034 C and 100 mg. for 1056 C. Preparation 906 910-A which was supplied in solution doubled the weight of adrenals in the test animals when a total of 5 cc. was injected.

TABLE 1. SUMMARY OF EXPERIMENTS ON THE INDUCTION OF LACTATION IN HYPOPHYSECTOMIZED GUINEA PIGS RECEIVING VARIOUS COMBINATIONS OF PITUITARY AND ADRENAL CORTICAL SUBSTANCES

No. of Animals	Days Initial Treatment	Initial Daily Treatment			Lactation Resulting	Subsequent Control Treatment			Lactation Resulting
		Lactogen mg.	DCA mg.	Comp. E mg.		Lactogen mg.	Cort. Ext. cc.	Adrenotrophin mg.	
		Experiments with lactogen 096910 A;[1] Series 1							
2	7	2.0			0, 0	2.0	2.0		3+, 4+
1	7	2.0	2.0		0	2.0	2.0		4+
4	5	3.0			1+, 1+,[3] 2+, 2+	3.0	2.0		3+, 4+
1	7	2.0	2.0		1+	2.0	2.0		4+
2	7	3.0	0.5		1+, 1+				
2	7	2.0		1.0	2+, 3+				
		Experiments with lactogen 1034 C; Series 2							
2	6	3.0			0, 0				
3	6	6.0			0, 0, 0[2]	6.0	2.0		3+, 3+, 4+
3	6	3.0	1.25		0, 0, 0	3.0	2.0		4+, 4+, 3+
2	6	6.0	1.25		0, 0	6.0	2.0		4+, 4+
		Experiments with lactogen 1056 C alone; Series 3							
5	5–8	3.0			0, 0, 0,[2] 0, 0	3.0	3.0		3+, 2+, 3+
3	6–8	4.5			0, 0, 0	4.5	2.0		4+, 4+, 4+
2	6	6.0			0, 0	6.0	2.0		3+, 4+
2	6	6.0			0, 0	6.0		10.0	4+, 4+
3	6	6.0			0, 0, 0	10.0		10.0	3+, 4+, 4+
2	6	10.0			1+, 1+	10.0	2.0		4+, 4+
		Experiments with lactogen 1056 C+DCA; Series 4							
2	6	3.0	1.0		0, 0				
2	7	4.5	1.0		0, 0	4.5		10.0	3+, 4+
2	7	3.0	2.5		0, 0[2]	3.0	2.0		4+, 4+
4	5–7	4.5	2.5		0, 0, 0, 0[2]				
2	10	4.5	4.0		0, 0	4.5	2.0		4+, 4+
2	7	6.0	1.0		0, 0	6.0			0, 0
1	7	6.0	1.0		0[2]	6.0	2.0		4+
2	8	10.0	2.5		0, 0	10.0			1+, 1+
		Experiments with lactogen 1056 C+Comp. E; Series 5							
2	8	3.0		1.0	0,[2,3] 0[2]				
1	8	4.5		1.0	0	4.5		13.0	4+
2	7	3.0		1.0	2+,[2] 3+				
2	8	4.5		1.0	2+, 3+	4.5		13.0	4+, 4+
5	3–8	3.0		1.0	1+, 2+, 2+, 3+, 3+				

[1] Dry weight not known. Dose in cc.
[2] Mammary glands inadequately developed for good response.
[3] Had pituitary fragment at autopsy.

lactogenic preparation, and did not induce lactation when given alone in doses as high as 6 mg. daily. The responses were similarly negative when the lactogen was given together with desoxycorticosterone acetate. When the lactogen was supplemented with cortical extract, however, a copious lactation occurred in every case.

Experiments with lactogen 1056C. This also was a highly purified material and only with 10 mg. daily doses could any suggestion of lactation be obtained when it was given alone (Series 3). The addition of either cortical extract or adrenotropin to the lactogenic therapy induced a good lactation.

With this lactogen, given in doses from 3 to 10 mg. daily, extensive tests were made of the possible supplemental action of desoxycorticosterone acetate (Series 4). The latter was given in doses of from 1 to 4 mg. daily. Unlike treatment with cortical extract or adrenotropic hormone, administration of desoxycorticosterone did not permit the induction of lactation in any case. The fact that 10-mg. daily doses of the lactogen induced scant lactation as noted above when given alone, but did not do so when combined with 2.5 mg. doses of desoxycorticosterone suggested that the latter actually inhibited lactation.

In 12 cases (Series 5) the effects of supplemental therapy with 17-hydroxy-11-dehydrocorticosterone were studied when given in combination with lactogen 1056C. Since only small amounts of this adrenal compound were available, only 1-mg. daily doses were used. The results, although not dramatic, stood in marked contrast to those with desoxycorticosterone. Lactation was induced in 10 of the 12 cases. It was, however, not maximal in its extent, and was not as great as the response obtained with 2 cc. of the cortical extract used or with 10 mg. of adrenotropic substance. Probably larger doses would have given a better response, but that remains to be determined.

Inhibition of lactation with desoxycorticosterone acetate. The results of these experiments seemed to show that desoxycorticosterone acetate is not able to serve as the adrenal cortical factor which is necessary for lactation in the hypophysectomized guinea pig. Indeed, they suggested that it actually may have exerted an inhibiting influence. This possibility was put to test by preparing a group of animals for lactation by treatment for 25 or 30 days with estrogen. None of these animals was hypophysectomized nor was lactogenic hormone injected.

In 11 instances treatment with estrogen was withdrawn and the animals observed daily. As was anticipated, each lactated within 5 days. Five of these animals received no further treatment, and lactation continued for 10 or 12 days. In the remaining 6 cases desoxycorticosterone acetate, 3.0 mg. daily, was given for 10 days. Lactation ceased in each instance within 3 days, but recurred when desoxycorticosterone acetate was withdrawn.

Nine animals received desoxycorticosterone acetate, 3.0 mg. daily,

beginning immediately after treatment with estrogen was stopped, and continued for 8 to 12 days. None of these lactated during treatment with desoxycorticosterone acetate, but in each instance fair to good lactation occurred within 4 days after desoxycorticosterone acetate was withdrawn. In none of these animals was the average lactation as good as would have been expected after the cessation of treatment with estrogen, but the qualitative response appeared similar in every respect.

Nine animals were injected with 2.0 mg. daily and four with 1.0 mg. daily of desoxycorticosterone acetate for 8 to 12 days beginning immediately after treatment with estrogen was withdrawn. Six of the animals which received 2.0 mg. daily did not lactate during treatment while the remaining 3 on that dose and the 4 which received 1.0 mg. daily showed scanty to fair lactation for at least part of the period of treatment with desoxycorticosterone acetate. Lactation in each of these animals improved when desoxycorticosterone acetate was withdrawn.

These experiments indicated that desoxycorticosterone acetate inhibited lactation, but gave no real clue as to the route of inhibition. This might occur as a direct effect on the mammary glands or through an inhibition of the production or release of lactogenic hormone. An attempt was made to evaluate these possibilities. Ten male guinea pigs were injected with 20 R.U. of estrogen daily for 25 days. This treatment was then withdrawn. Four animals received 2.5 mg. of desoxycorticosterone acetate daily for 8 days following which period they received the same dose of desoxycorticosterone acetate with the addition of 10 mg., daily of lactogen (1056C) for 6 to 8 days. In each instance good lactation occurred within 3 days after the initiation of treatment with lactogen. Four animals received both desoxycorticosterone acetate and lactogen immediately after treatment with estrogen was withdrawn. In each case good lactation occurred within 5 days. Two animals received no treatment after the withdrawal of estrogen. They lactated 4 days later. This evidence indicates that desoxycorticosterone acetate probably exerts its lactation-inhibiting effect, in part at least, by suppressing the production or release of lactogen from the hypophysis.

Mammary growth induction with desoxycorticosterone acetate. Since other steroid hormones which inhibit lactation also promote mammary development the results seen in animals treated with desoxycorticosterone acetate suggested that a similar relation might be expected. That such an action does occur in the mouse had been shown by van Heuverswyn, Folley and Gardner (1939).

As a test of this relation in the guinea pig, 8 young male animals were injected with 2 mg. of desoxycorticosterone acetate daily for 25 to 40 days. In each instance evidence of stimulation was visible in the growth of the nipples after the seventh day. Mammary biopsies were

taken in 4 of these animals at the time treatment was stopped. Appreciable development, qualitatively similar to that seen in estrogen-treated animals, had occurred in each instance. All 8 animals lactated within 4 days after the cessation of treatment. The secretion varied from scanty to fairly good and continued for about 6 or 7 days.

Four males received 1 mg. of desoxycorticosterone acetate daily for 30 days. Mammary growth was not as marked at this level and only 2 animals lactated when treatment was stopped.

Pellets of desoxycorticosterone acetate, weighing 25 mg. each, were implanted into 4 males. No growth of the nipples occurred over a period of 5 weeks and tissue from the mammary area showed that no growth had been induced. The pellets were recovered, their weight in each instance being approximately 20 mg. Since only 5 mg. were absorbed, the absence of mammary growth in these animals was not surprising.

DISCUSSION

The results present further evidence of the important rôle played by the adrenal cortex in the initiation and maintenance of lactation. Very potent preparations of lactogenic hormone are powerless to induce the secretion of milk in hypophysectomized guinea pigs unless aided by the action of adrenal substances. This aid may be provided with equal effectiveness in the form of hormone produced in the adrenal cortex as the result of stimulation by adrenotropic hormone or directly as cortical hormone supplied by injection.

The interpretation of the varied and heterogenous group of experiments reported here, together with those from other sources, demands recognition of several basic points. These include the following.

a). The lactogenic hormone will not, unaided, induce lactation in the absence of the hypophysis, but preparations of this hormone usually are contaminated with the adrenotropic factor and if given in large enough doses will initiate lactation. Thus, 2 of the 3 preparations used in this study, 096 910 A and 1056 C, induced lactation at the higher levels of dosage.

b). Some substance or substances produced in the adrenal cortex are essential for the secretion of any sizable quantity of milk in both the guinea pig and the rat, and they seem to be more vitally necessary in the guinea pig. These substances probably act, in part at least, through mechanisms involving both of the well-known functions of the adrenal cortex, *i.e.*, on salt and water, and on carbohydrate metabolism.

The fact that a proper control of electrolyte metabolism can be helpful, although not self-sufficient, in the repair of the lactation deficiencies due to adrenal dysfunction is suggested by three lines of evidence: *a*) it was observed in earlier studies that a scanty lactation could be induced in the hypophysectomized, lactogen-treated guinea pig if a high salt diet were given (Nelson and Gaunt, 1937a, b); *b*) in

the adrenalectomized rat, desoxycorticosterone, which has little effect on carbohydrate metabolism, but a marked effect on electrolyte metabolism, enhances milk secretion although it does not raise it to a normal level (Gaunt, Eversole and Kendall, 1942); and *c*) experimental alterations in electrolyte and water balance affect mammary function adversely (Nelson and Gaunt, 1937a, b; Levenstein, 1937).

Gomez and Turner (1936, 1937) suggested that disturbances in carbohydrate metabolism were also important causes of the lactation deficiencies seen in hypophysectomized animals. The importance of these disturbances is further indicated by the failure of desoxycorticosterone, even in small doses which do not affect mammary growth, to permit the initiation of lactation in the guinea pig as reported here, whereas 17-hydroxy-11-dehydrocorticosterone, highly potent in affecting carbohydrate metabolism, will do so; and by the fact that in the adrenalectomized rat, the latter substance is the only one of the crystalline adrenal steroids which is known to maintain a normal lactation.

In addition to these factors the existence of more specific lactation-supporting substances of adrenal origin have been suggested (Brownell, Lockwood and Hartman, 1933: Spoor, Hartman and Brownell, 1933) and questioned (Gaunt and Tobin, 1936; Hurst, Meites and Turner, 1942).

c). Substances which promote mammary growth, in general, inhibit lactation. Thus estrogenic hormone, a most potent stimulant to the growth of the mammary gland, inhibits secretion of milk. The same appears to be true, although from a quantitative standpoint to a lesser degree, of desoxycorticosterone. Such inhibition is probably effected in the hypophysectomized animal by a direct action on the mammary gland, and, in the normal animal by an effect on the hypophysis as well as the mammary gland. This mechanism has been demonstrated for estrogenic hormone (Nelson, 1937).

The guinea pig is apparently highly sensitive to these lactation-inhibiting effects of desoxycorticosterone, and, therefore, in this species a lactation-supporting action might be expected only with small doses, if at all. The rat is more resistant to the lactation-inhibiting action of this steroid (Gaunt, Eversole and Kendall, 1942; Folley, 1942), and therefore in this species a lactation-supporting rôle can be demonstrated.

With these considerations in mind most of the observations now available on this subject can be harmonized.

SUMMARY

Previous findings that lactation could not be induced in hypophysectomized guinea pigs by lactogenic hormone alone, but could be initiated if adrenotropic hormone or adrenal cortical extract were given with the lactogen, were confirmed and extended.

Lactation could not be induced in hypophysectomized guinea pigs when desoxycorticosterone acetate was given together with adequate amounts of lactogenic hormone.

Lactation could be induced when 17-hydroxy-11-dehydrocorticosterone was given in such cases with lactogenic hormone.

It was suggested in hypophysectomized animals and demonstrated in intact animals that desoxycorticosterone acetate (3 mg. daily) has a lactation-inhibiting influence. This was probably related to the fact that mammary growth could be induced by this steroid when it was administered in 2-mg. daily doses.

REFERENCES

BROWNELL, K. A., J. E. LOCKWOOD AND F. A. HARTMAN: *Proc. Soc. Exper. Biol. & Med.* 30: 783. 1933.

FOLLEY, S. J.: *Nature* 150: 266. 1942.

GAUNT, R., W. J. EVERSOLE AND E. C. KENDALL: *Endocrinology* 31: 84. 1942.

GAUNT, R., AND C. E. TOBIN: *Am. J. Physiol.* 115: 588. 1936.

GOMEZ, E. T., AND C. W. TURNER: *Proc. Soc. Exper. Biol. & Med.* 35: 365. 1936.

GOMEZ, E. T., AND C. W. TURNER: *Proc. Soc. Exper. Biol. & Med.* 36: 78. 1937.

VAN HEUVERSWYN, J., S. J. FOLLEY AND W. U. GARDNER: *Proc. Soc. Exper. Biol. & Med.* 41: 389. 1939.

HURST, V., J. MEITES AND C. W. TURNER: *Proc. Soc. Exper. Biol. & Med.* 49: 592. 1942.

LEVENSTEIN, I.: *Anat. Rec.* 67: 477. 1937.

NELSON, W. O.: *Am. J. Anat.* 60: 341. 1937.

NELSON, W. O., AND R. GAUNT: *Proc. Soc. Exper. Biol. & Med.* 34: 671. 1936.

NELSON, W. O., AND R. GAUNT: *Proc. Soc. Exper. Biol. & Med.* 36: 136. 1937a.

NELSON, W. O., AND R. GAUNT: *Cold Spring Harbor Symp. Quant. Biol.* 5: 398. 1937b.

SPOOR, H. J., F. A. HARTMAN AND K. A. BROWNELL: *Am. J. Physiol.* 134: 12. 1941.